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Caractérisation génétique et phénotypique de *Cryptosporidium* : de la souris à l'homme

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5) **Osman *et al*, 2014**. **14ème journée André Verbert**, Lille, France. (11 Septembre 2014). Prévalence de *Giardia duodenalis* et *Cryptosporidium* spp. et facteurs de risque chez des écoliers à Tripoli

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10) **Osman *et al*, 2013**. **3ème Forum Doctoral-EDST-UL**, Beyrouth, Liban (25 Juin 2013). Caractérisation génétique et phénotypique de *Cryptosporidium*: de la souris à l'homme.

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5) **Notions fondamentales en statistiques incluant spécificité petits échantillons – Université Lille 2 Droit et Santé.** Lille, France. (12 – 15 Mai 2014).

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Sommaire

Liste des abréviations……..15
I. Résumé……..17
II. Abstract…….....19
III. Introduction……..21
IV. Généralités……25
1. Pathogenesis of *Cryptosporidium* in humans ……26
1. Pathogen……..26
1.1. History……..26
1.2. Life cycle……..27
1.3. *Cryptosporidium* species……...31
1.4. Genomics of *Cryptosporidium* species……34
2. Epidemiology……...37
3. Clinical features……...41
3.1. Clinical manifestations associated to infecting *Cryptosporidium* species…....43
3.2. *Cryptosporidium* and cancer (some clinical evidences)……...44
4. Pathogenesis and Immunity……45
4.1. Adherence to and invasion of epithelial host cells……...46
4.2. Epithelial cellular processes initiated by *Cryptosporidium* infection……..48
4.3. *Cryptosporidium* and cancer (some experimental evidences)……51
5. Diagnosis……53
5.1. Staining methods……53
5.2. Immunological methods……..54
5.3. Molecular tools……57
6. Treatment……60
7. Control and prevention……...61
8. References……..63
2. Epidémiologie moléculaire de la cryptosporidiose au Moyen Orient…..……80
3. *Cryptosporidium* et cancer……97
V. Objectifs et Stratégies……...104
VI. Résultats……107
1. Axe 1 : Premières données d'épidémiologie moléculaire et facteurs de risque liés à l'infection par *Cryptosporidium* spp. au Liban…….....108
1. Article 1 : Initial data on the molecular epidemiology of cryptosporidiosis in Lebanon……...109

2. Article 2 : Prevalence and Risk Factors for Intestinal Protozoan Infections with *Cryptosporidium*, *Giardia*, *Blastocystis* and *Dientamoeba* among Schoolchildren in Tripoli, Lebanon……124

2. Axe 2 : Etude de la prévalence de *Cryptosporidium* spp. dans les échantillons animaux et l'évaluation du pouvoir zoonotique du parasite……..157

1. Article 3 : Cryptosporidiosis in humans and cattle in a rural area of Northern Lebanon……..158
2. Article 4 : Prevalence and genetic diversity of the intestinal parasites *Blastocystis* sp. and *Cryptosporidium* spp. in household dogs in France and evaluation of zoonotic transmission risk…….171
3. Prévalence et caractérisation moléculaire de *Cryptosporidium* chez plusieurs groupes d'animaux des parcs zoologiques français……..193
4. Article 5 : Identification of *Cryptosporidium* species in fish from Lake Geneva (Lac Léman) in France……210

3. Axe 3 : Etude de la pathogénicité de *Cryptosporidium* spp.……...……..235

1. Article 6 : High Association of *Cryptosporidium* infection with Digestive Cancer in Lebanese patients……236
2. Article 7 : *Cryptosporidium parvum*-induced ileo-caecal adenocarcinoma and WNT signaling in a rodent model…….255

VII. Discussion……...…285

VIII. Conclusions et Perspectives……..301

IX. Annexe……305

1. Travaux présentés aux congrès scientifiques…….…….……..306

2. Autres articles publiés dans des journaux internationaux…….……..……...320

1. Article A : Short report: Molecular epidemiology of *Blastocystis* in Lebanon and correlation between subtype 1 and gastrointestinal symptoms……...320
2. Article B : Acute *Blastocystis*-Associated Appendicular Peritonitis in a Child, Casablanca, Morocco……..……331
3. Article C : Draft genome sequence of the intestinal parasite *Blastocystis* subtype 4-isolate WR1…….339

X. Références bibliographiques……345

Liste des abréviations

ADN	Acide Desoxyribonucléique
AEECL	Association Européenne pour l'Étude et la Conservation des Lémuriens
ANOFEL	Association française des enseignants et praticiens hospitaliers titulaires de parasitologie et mycologie médicale
APC	Adenomatous Polyposis Coli
ARNr 18S	Acide Ribonucléique de la petite sous unité ribosomique
ARNt	ARN de transfert
ATP	Adénosine triphosphate
CCR	Cancer colorectal
C. hominis	*Cryptosporidium hominis*
C. parvum	*Cryptosporidium parvum*
C. muris	*Cryptosporidium muris*
DAPI	4',6'-diamidino-2-phénylindole
EBV	Epstein barr virus
ELISA	Enzyme Linked ImmunoSorbent Assay
HAART	Highly active antiretroviral therapy ; Traitement antirétroviral hautement actif
HSP	Heat shock protein
IARC	International Agency for Research on Cancer
IBS	Irritable Bowel Syndrome ou Syndrome du Côlon Irritable
Ig	Immunoglobuline
IL	Interleukine
IMDM	Iscove's modified Dulbecco's medium
ITS	Internal Transcribed Spacer
kb	Kilobase
pb	Paires de bases
MLO	Mitochondrion-Like Organelle

MET: Microscopie électronique à transmission

NFκB : Facteur nucléaire κB

OMS	Organisation Mondiale de la Santé
PI3K	Phosphatidyl inositol 3- kinase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
SCID	Severe combined immunodepression (sans traitement à la dexaméthasone)

SCID-D	Severe combined immunodepression (avec traitement à la dexaméthasone)
SIDA	Syndrome de l'immunodéficience acquise
PI-IBS	IBS post-infectieux
RFLP	Restriction Fragment Length Polymorphism
TRAP	Thrombospondin-related adhesive protein
VHB	Virus de l'hépatite B
VHC	Virus de l'hépatite C
VIH	Virus de l'immunodéficience humaine
sp.	Espèce
ssrRNA	Small subunit ribosomal RNA
ST	Sous-type

I. Résumé

Les parasites du genre *Cryptosporidium* comprennent des espèces infectant le tractus gastro-intestinal ou respiratoire d'un grand nombre de vertébrés y compris l'homme. Ces protistes intracellulaires sont les agents d'une zoonose cosmopolite à transmission oro-fécale, la cryptosporidiose. Au vu des travaux réalisés dans notre laboratoire, nous savons à présent que *Cryptosporidium parvum* est également capable d'induire des néoplasies digestives chez un modèle murin SCID (Severe Combined Immunodeficiency mice), traitées ou pas par la dexaméthasone. Alors que *C. muris*, une autre espèce de *Cryptosporidium*, induit une infection chronique non associée à des transformations néoplasiques.

Pour toutes ces raisons, il nous est apparu intéressant d'effectuer un travail de thèse articulé autour de trois axes principaux, l'épidémiologie, la transmission et la pathogénicité du parasite *Cryptosporidium*. Nous nous sommes intéressés dans un premier temps à l'épidémiologie moléculaire et la biodiversité génétique de *Cryptosporidium* dans des populations humaines de la région du Nord-Liban. Ceci nous a permis de mettre en évidence une prévalence de 5% de *Cryptosporidium* chez la population générale avec une prédominance de *C. hominis*. Ce qui constituait les premières données épidémiologiques de la cryptosporidiose au Liban. Ensuite d'autres études nous ont permis de montrer que cette prévalence pouvait atteindre même 10% chez les patients symptomatiques et les enfants.

Dans un second temps, nous avons voulu étudier le mode de transmission du parasite et les facteurs de risque pouvant y être associés. Pour ce faire, une recherche du parasite a été réalisée aussi bien au Liban qu'en France chez des animaux d'élevage, sauvages, de compagnie et en captivité. Une première étude a été réalisée chez des patients et des bovins du Nord-Liban. L'ensemble des données rapportées nous permettent de suggérer un mode de transmission de la cryptosporidiose majoritairement anthroponotique au Liban, mais les résultats du génotypage ne permettent pas d'exclure la présence d'une transmission zoonotique. D'autres études réalisées en France, notamment sur des échantillons de selles collectées auprès des zoos de la Palmyre (à Royan, Charente-Maritime) et de Lille (Nord de la France) ont montré un taux de prévalence de Cryptosporidium spp inférieur à 1%. Ces animaux ne semblent donc pas être un réservoir potentiel de cette infection. Alors que chez les poissons sauvages du Lac Léman (France), nous avons pu identifier la présence entre autre de l'espèce zoonotique *C. parvum* dans l'estomac et l'intestin des poissons. Ceci nous permet de considérer les poissons comme étant une source de contamination potentiel pour l'homme, l'animal mais également pour l'environnement.

Enfin le troisième axe avait pour but d'étudier la pathogénicité de ce parasite. Pour commencer nous avons voulu étudier l'association entre la pathologie cancéreuse et le parasitisme par *Cryptosporidium* chez l'homme. Une recherche du parasite a donc été réalisée dans des biopsies coliques et gastriques inclues en paraffine appartenant à des patients atteints ou non de cancers digestifs. Une différence significative a été rapportée entre la prévalence de la cryptosporidiose retrouvée chez la population de patients présentant des lésions cancéreuses (17%) et celle du groupe control constitué de patients non cancéreux mais présentant des symptômes (7%) *p-value* = 0.03. Ensuite nous avons voulu explorer les mécanismes de la cancérogénèse induite par la souche IOWA de *C. parvum* au niveau de la région iléocæcale des souris SCID traitées par la dexaméthasone. Pour ce faire nous nous sommes intéressés à quatre marqueurs de voies de signalisation cellulaires impliquées dans la survenue de cancers colorectaux (APC, Bêta-caténine, P53 et K-ras). Nous avons ainsi pu montrer que la voie Wnt était impliquée dans ce processus. L'ensemble de ces données obtenues chez l'animal et chez l'homme montre que ce parasite a un impact important en santé humaine et animale.

II. Abstract

Parasites of the genus *Cryptosporidium* comprise species infecting the gastrointestinal or respiratory tract of a wide variety of vertebrates including humans. These intracellular protists are the agents of a cosmopolitan zoonosis, with féco-oral transmission, cryptosporidiosis. Recent work from our laboratory, showed that the zoonotic species *Cryptosporidium parvum* is capable to induce digestive neoplasia in a SCID Severe Combined Immunodeficiency Mice (SCID) model, treated or not with dexamethasone. However *C. muris*, another species of *Cryptosporidium*, induces chronic infection in this rodent model but is not associated with neoplastic transformation.

For all these reasons, it seemed interesting to carry out a thesis project articulated around three different axes: epidemiology, transmission and pathogenesis of the *Cryptosporidium* infection. We focused initially on the molecular epidemiology and genetic biodiversity of this parasite among human populations in North Lebanon. We found a *Cryptosporidium* prevalence of 5% among the general population, being *C. hominis* the predominant species. This prevalence could reach until 10% in symptomatic patients and children. This is the first epidemiological data about cryptosporidiosis in this country.

Secondly, we studied the transmission routes and the main risk factors associated with the transmission of this parasite. To do this, a first study was conducted in parallel among animal populations in North Lebanon and France. The reported data suggest a predominance of an anthroponotic route of transmission for cryptosporidiosis in Lebanon, but the results of genotyping does not exclude the presence of zoonotic transmission. Other studies conducted in France, especially based on collection of stool samples in the zoos of Palmyre (Royan, Charente-Maritime) and Lille showed that *Cryptosporidium* spp were present in less than 1% of captivity animals. The low prevalence strongly demonstrates that these animals play a negligible role as potential reservoirs of infection. While in wild fish, we could identify the presence of *C. parvum*, a zoonotic species, in the stomach and the gut of fish. These data suggest that the fish could be a natural host of *C. parvum* and a potential source of contamination for humans, animals but also for the environment.

The third topic aimed to study the pathogenicity of this parasite. Firstly, we wanted to study the association between digestive cancer and parasitism with Cryptosporidium in humans. Cryptosporidium molecular detection was therefore carried out in colonic and gastric biopsies belonging to patients with and without digestive cancers of recent diagnosis collected in North

Lebanon. A statistically significant difference was observed between the prevalence of cryptosporidiosis found among the population of patients with digestive cancer (17%) and that of the control group consisting of non-cancer patients but with digestive symptoms (7%) (p-value = 0.03). All these data obtained in animals and humans strengthens the importance of this parasite in public health. Finally, we explored metabolic pathways potentially involved in the development of C. parvum-induced ileo-caecal oncogenesis in the SCID model treated with dexamethasone. We searched for alterations in genes or proteins commonly involved in cell cycle, differentiation or cell migration, such as β-catenin, Apc, E-cadherin, Kras and p53. We were able to show that the Wnt pathway was involved in this process.

III. *Introduction*

La cryptosporidiose est une zoonose opportuniste cosmopolite causée par diverses espèces appartenant au genre *Cryptosporidium*. Un Apicomplexa qui se multiplie dans les cellules épithéliales du tractus gastro-intestinal et du système respiratoire d'un grand nombre de vertébrés, y compris l'homme (Chalmers and Katzer, 2013; Ryan, Fayer, and Xiao, 2014).

Les oocystes sont la forme de résistance et de dissémination du parasite. Le mode de transmission de cette infection est oro-fécal, soit indirectement par ingestion d'oocystes contaminants l'eau ou les aliments soit par contact direct avec un sujet infecté. Ce dernier peut être un homme ou un animal, la maladie peut donc se répandre par la voie anthroponotique ou zoonotique (Chalmers and Katzer, 2013; Ryan, Fayer, and Xiao, 2014). Actuellement, plus d'une vingtaine d'espèces ont été validés pour le genre *Cryptosporidium*. Les espèces qui parasitent le plus fréquemment l'homme sont *Cryptosporidium parvum* (*C. parvum*) et *C. hominis*, même si des infections par *C. meleagridis*, *C. cuniculus*, *C. andersoni*, *C. felis*, et *C. canis* ont été rapportées mais le plus souvent chez des patients à risques (Chalmers and Katzer, 2013; Xiao, 2010).

Cette parasitose émergente a un impact considérable chez le patient immunodéprimé, chez qui elle peut devenir chronique voire létale. L'infection est toutefois possible chez les sujets immunocompétents, de façon sporadique ou associée à des épidémies, mais cela se traduit par des diarrhées auto-résolutives, généralement sans complications. *Cryptosporidium* est à l'origine de plus de 165 épidémies d'origine hydrique (Chalmers, 2012). La plus importante fut celle de Milwaukee (Wisconsin, États-Unis) en 1993, qui contamina près de 403 000 personnes via le réseau de distribution d'eau (Mac Kenzie et al., 1994). De plus, des données récentes ont montré que *Cryptosporidium* est parmi les principales causes de diarrhée infantile modérée à sévère chez les enfants âgés de moins de 2 ans dans les pays en développement (Kotloff et al., 2013; Striepen, 2013). De même, selon l'Organisation Mondiale de la Santé (OMS), la cryptosporidiose est inclue dans la liste des maladies négligées qui constituent un frein au développement socio-économique dans les pays en voie de développement et pour lesquelles des études de terrain sont indispensables à leur compréhension et à l'établissement de conduites permettant leur contrôle (Savioli, Smith, and Thompson, 2006). Cette maladie est un véritable problème de santé publique.

Le Liban, à l'instar de la plupart des pays en développement, est très touché par les parasitoses intestinales (Hamze et al., 2004; Hamze, Naja, and Mallat, 2008). Malgré la virulence de ce protiste intracellulaire et son impact socio-économique et en santé publique

non négligeable, aucune information n'était disponible concernant la situation de la cryptosporidiose dans ce pays avant ce travail de thèse. Ces informations sont indispensables afin que ce pays puisse définir des stratégies de prévention, de contrôle et de lutte contre cette parasitose. La France, par contre, semble peu touchée par la cryptosporidiose (ANOFEL, 2010). Cependant, peu d'études ont été réalisées sur des populations animales autres que les animaux d'élevages (Follet et al., 2011; Rieux et al., 2013; Rieux et al., 2014).

D'autre part, l'implication de *C. parvum* dans l'induction de néoplasies digestives a été démontrée en 2007 dans le laboratoire Biologie et Diversité des Pathogènes Eucaryotes Emergents (BDPEE) chez un modèle murin d'infection chronique (i.e. le modèle souris SCID traité ou non à la Dexaméthasone) (Certad et al., 2007). Il a ainsi pu être mis en évidence que l'infection par *C. parvum* induisait chez ces souris, le développement d'adénocarcinomes digestifs invasifs (Benamrouz et al., 2012a; Benamrouz et al., 2012b; Certad et al., 2012; Certad et al., 2010a; Certad et al., 2010b). Cependant, *C. muris*, une autre espèce de *Cryptosporidium*, n'a pas induit ce type de transformation épithéliale et les raisons de ces différences dans l'expression de la maladie restent inconnues (Certad et al, 2007).

L'ensemble de ces données expérimentales chez l'animal ainsi que d'autres données clinico-épidémiologiques chez l'homme suggère fortement que *Cryptosporidium* pourrait avoir vraisemblablement un pouvoir carcinogène qui s'exprime dans le tractus gastro-intestinal.

Avant de présenter en détail les différents résultats obtenu durant ma thèse, je commencerai tout d'abord par faire un état de l'art de ce que l'on sait sur *Cryptosporidium*, son épidémiologie et enfin sa pathogénicité. Ce chapitre, nommé « Généralités », bien que non exhaustif permettra :

- Dans un premier temps, de donner une vue d'ensemble de ce qu'est *Cryptosporidium* et son impact en santé humaine. Pour ce faire, j'ai intégré à cette partie un chapitre de livre intitulé « Pathogenesis of *Cryptosporidium* in Humans » que j'ai rédigé avec d'autres membres de mon laboratoire d'accueil Français. Ce dernier aborde notamment, la biologie du parasite, les manifestations cliniques et la réaction immunitaire qu'il induit chez son hôte, ses facteurs de virulences, les outils de diagnostiques ainsi que les traitements dont nous disposons à l'heure actuelle pour lutter contre cette maladie.

- Dans un second temps, de dresser un état des lieux des connaissances actuelles concernant l'épidémiologie de *Cryptosporidium*. En effet, l'étude de l'épidémiologie du parasite est nécessaire à une meilleure connaissance de sa diversité génétique et à une meilleure compréhension de son mode de transmission et ainsi à l'établissement de moyens de prévention. C'est pourquoi, j'ai intégré au manuscrit, un travail bibliographique qui rassemble les données publiées sur l'épidémiologie de *Cryptosporidium* dans divers pays du globe et plus particulièrement au Moyen Orient.

- Et enfin, dans un troisième temps, cet état de l'art me permet de revenir sur une découverte faite dans mon laboratoire d'accueil Français, qui a permis de mettre en évidence pour la première fois que l'espèce *C. parvum* était dotée d'un pouvoir carcinogène.

IV. *Généralités*

1. Pathogenesis of *Cryptosporidium* in humans

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1. Pathogen

1.1. History

The genus *Cryptosporidium* is a member of the phylum *Apicomplexa*. It is composed of protozoan parasites that infect epithelial cells in the microvillus border of the gastrointestinal tract of all classes of vertebrates, including humans. *Cryptosporidium* was described for the first time by Ernest Tyzzer in 1907 who isolated the parasite from the gastric mucosa of mice (*Mus musculus*) and named *Cryptosporidium muris (C. muris)* (Tyzzer, 1910). In 1912, E. Tyzzer described again this parasite but this time in the small intestine of mice. Smaller in size than the parasite described in the stomach, it looked like a different species that was named *Cryptosporidium parvum* (*C. parvum*) (Tyzzer, 1912). It was not until the 1970s that first cases of human cryptosporidiosis in patients with severe watery diarrhea were reported (Nime et al., 1976). However, it is only in the early 1980's that clinical significance and pathogenicity of this parasite were recognized, especially due to a high morbidity and mortality found in immunocompromised patients (Tzipori and Widmer, 2008). In particular, at this time cryptosporidiosis emerged as a frequent cause of diarrhea in HIV/AIDS patients, and the link with AIDS was so strong that the infection became one of the defining features of the syndrome (Colford et al., 1996; Mwachari et al., 1998).

Cryptosporidium has been also considered as the infectious agent responsible of more than 60% of waterborne outbreaks (Baldursson and Karanis, 2011). The best known example of a waterborne outbreak caused by this parasite was described in the spring of 1993 in Milwaukee (United States of America) due to increased contamination of source water and a breakdown in the water filtration process at a water treatment plant in the city (Naumova et al., 2003). This outbreak affected more than 400,000 people who drank tap water contaminated with oocysts (Mac Kenzie et al., 1994). The resistance of *Cryptosporidium* oocysts in water and the environment, the lack of treatment or vaccination in humans and animals, as well as its socio-economic implications, have led the World Health Organization (WHO) to include this parasite in the neglected disease initiative of the WHO (Savioli, Smith,

and Thompson, 2006) and to use *Cryptosporidium* as a "reference pathogen" for the faecal-orally transmitted protozoa in the design and implementation of WHO Guidelines for Drinking Water Quality (Medema, 2009; WHO, 2011). Monitoring the presence of oocysts in water is now part of the surveillance to support water safety plans (WHO, 2011).

The final step in our evolving perception of the public health significance of *Cryptosporidium* parasites has been the inclusion of *C. parvum* and *C. hominis* in the list of category B infectious agents due to their bioterrorism potential (Widmer and Sullivan, 2012). In fact, the low infectious dose, the resistance of the oocyst to many disinfectants and the potentially severe symptoms caused by the parasite could motivate the malicious contamination of centralized water supply systems with *Cryptosporidium* oocysts.

1.2. Life cycle

The life cycle of *Cryptosporidium* begins with the ingestions of the sporulated, environmentally resistant oocysts by the susceptible host (Figure 1).

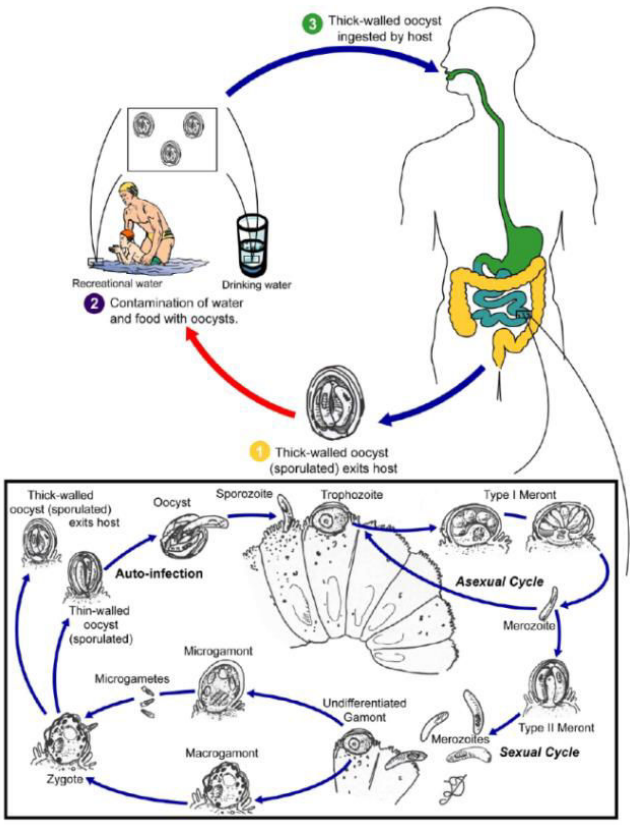


Figure 1. *Cryptosporidium* spp life cycle (Centers for Disease Control and Prevention)

Oocysts undergo excystation and release four infective sporozoites in the intestinal lumen upper small intestine. The excystation of oocysts has been reported to be triggered by various factors including temperature, pancreatic enzymes, bile salt, and carbon dioxide (Fayer and Leek, 1984; O'Donoghue, 1995; Reduker and Speer, 1985). After excystation, the released sporozoites penetrate the mucus layer and emerge to invade the microvilli at the brush border of the mucosal. The sporozoites possess an apical complex composed of micronemes, roptries and dense granules which are also present in other apicomplexa parasites and are involved in host cells attachment and invasion. Then a parasitophorous vacuole is formed around the parasite which develops into spherical trophozoite. An unusual feature of the parasitophorous vacuole is that it is located within the host cell plasma membrane, but outside the host cell cytoplasm, it is separated from the latter by a feeder organelle and cytoskeletal elements (Figure 2).

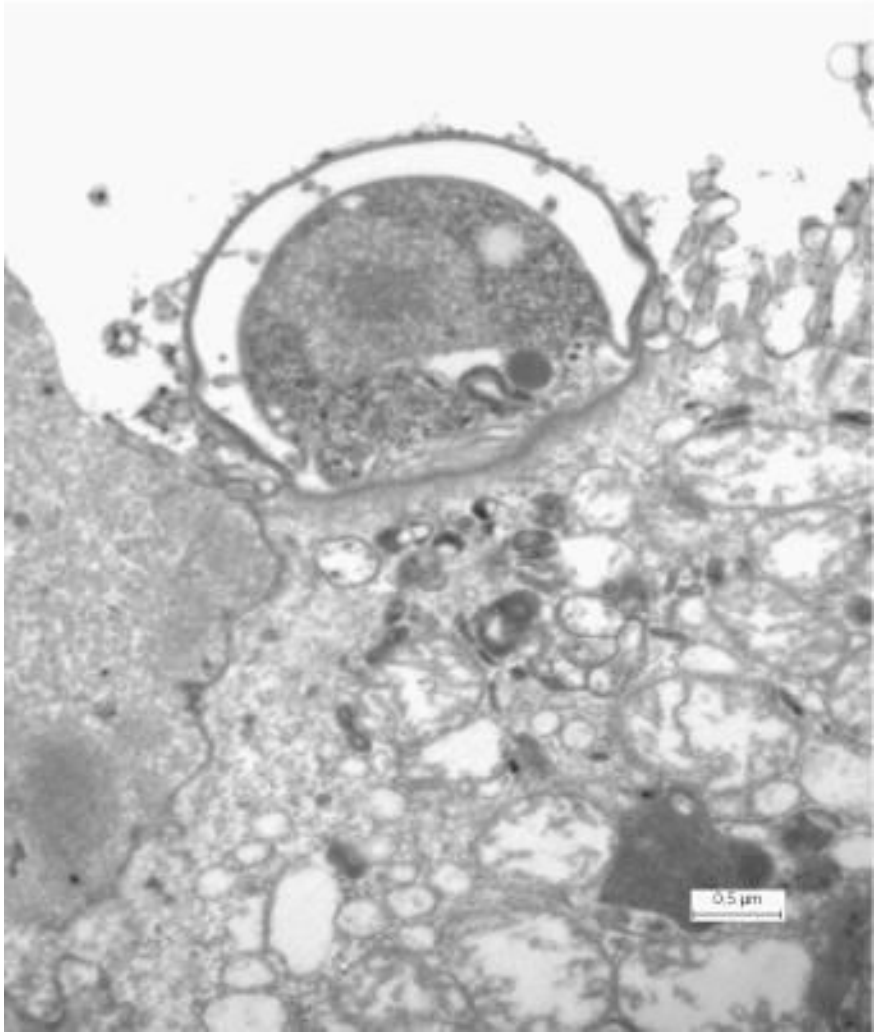
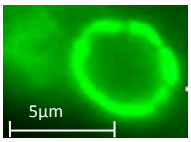
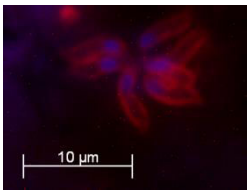
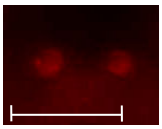
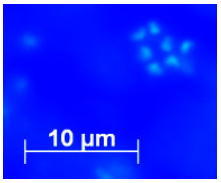
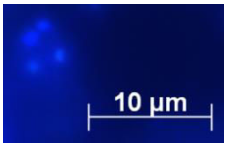


Figure 2. Transmission electron micrograph showing intracellular *Cryptosporidium* trophozoite inside their parasitophorus vacuole.

The trophozoites undergo asexual division (merogony) and form type I meronts containing 6 to 8 merozoites. Like sporozoites, merozoites escape the parasitophorous vacuole and attach again to the surface of epithelial cells, establishing amplified asexual infectious cycles. They undergo merogony once more and form either a further type I meront or a type II meront. The type II meront produce 4 merozoites which attach again to epithelial cells, but instead of developing into further meronts, they initiate gametogony. Individual merozoites produce either microgamonts or macrogamonts. Sixteen or more microgametes from the microgamont are released and each of these can fertilize a macrogamont to form a diploid zygote, which differentiates to an oocyst. Meiosis then results in the formation of four sporozoites. Most of the zygotes develop into oocysts with a thick-wall and are released into the lumen of the intestine, and then they are excreted into the environment. These thick-walled oocysts are immediately infective, allowing the spread of infection to other susceptible hosts. The minority develop into thin-walled oocysts which facilitate an auto-infective cycle that maintains infection without further ingestion of thick-walled oocysts (Chen et al., 2002). Characteristics of different *Cryptosporidium* life cycle stages can be observed in Table 1.

Table 1. Different life cycle stages of *Cryptosporidium*

Developmental stage	Form	Size (µm)	Number of nuclei	Image in immunofluorescence (marker)	Virulence factors in association with the developmental stage [a]
Oocyst	Spherical	4 - 6	4	 (Crypto Cel Reagent)	Excystation: Serine protease Aminopeptidase
Sporozoite	Banana shape	2-3 x 4-6	1	 (Sporo-Glo Cy3R and DAPI)	Adhesion: CSL Gp900 Gp60/40/15 P23 P30 TRAP-C1 Cp47 CpMUC1
Trophozoite	Round shape	1,5 - 2,5	1	 (Sporo-Glo Cy3R)	Invasion: Cp2 Cpa135 Phospholipase H4 CpSUB CpMuc
Type I meronte	Round shape	6 - 8	8	 (DAPI)	Multiplication and survival: CpABC CpATPase2 CpATPase3 HSP70 HSP90 Cysteine-protease Acetyl-co-synthetase
Type II méronte	Round shape	3 - 5	4	 (DAPI)	

[a] Data compiled from Okhuysen and Chappell, 2002; O'Hara and Chen, 2011; Bouzid et al., 2013.

1.3. *Cryptosporidium* species

To date, they are 26 validated species of *Cryptosporidium* spp. (Chalmers and Katzer, 2013; Slapeta, 2012). These species have been found to infect mammals, birds, reptiles, amphibians and fish. The two species that most commonly infect humans (>90%) are *C. hominis* and *C. parvum*, and while the former species seems to be mainly limited to humans, the latter has a wide range of hosts, including most major domestic livestock animal species (Fayer, 2010; Xiao, 2010). Other species that have less commonly been associated with human disease include *C. meleagridis*, *C. felis*, *C. canis*, and *C. cuniculus*. Some species of *Cryptosporidium* are also reported in sporadic human cases such as *C. andersoni*, *C. bovis*, *C. fayeri*, *C. muris*, *C. scrofarum*, *C. suis*, *C. tyzzeri*, *C. ubiquitum* and *C. viatorum* (Chalmers and Katzer, 2013). Some species of *Cryptosporidium* infect many host species, whereas others appear to be restricted to groups such as rodents or ruminants, and still others are known to infect only one host species. Some species primarily infect the stomach, whereas others primarily infect the intestine (Table 2).

Table 2. *Cryptosporidium* species and their association with human cryptosporidiosis

Cryptosporidium species	Reference	Mean oocyst dimensions (µm)	Major host(s)	Association with human cryptosporidiosis
C. muris	Tyzzer, 1907	7.0 x 5.0	Rodents	Rarely associated; individual reports from various developing countries
C. parvum	Tyzzer, 1912	5.0 x 4.5	Humans; mammals	Common in sporadic cases and outbreaks; cryptosporidiosis via zoonotic or anthroponotic transmission: directly or indirectly
C. meleagridis	Slavin, 1955	5.2 x 4.6	Birds; mammals	Infectivity data from experimental infections in adults indicate mild illness. Sporadic cases have been reported in Bangkok and Lima.
C. wrairi	Vetterling, 1971	5.4 x 4.6	Guinea pig	No association
C. bovis	Baker & Carbonell, 1974	4.9 x 4.6	Cattle	Rarely associated; cases reporting cattle contact in India and Australia.
C. cuniculus	Inman & Takeuchi, 1979 Robinson, 2010	5.6 x 5.4	Rabbit	Caused waterborne outbreak in UK; reports from France and children in Nigeria.
C. felis	Iseki, 1979	4.6 x 4.0	Cat	Epidemiologically associated to diarrhea in children in Peru; occasional sporadic cases in various countries including developed countries, especially immunocompromised persons and those with cat contact.
C. serpentis	Levine, 1980	6.2 x 5.3	Reptiles	No association
C. baileyi	Current, 1986	6.2 x 4.6	Chicken	No association

C. varanii	Pavlásek, 1995	4.8 x 4.7	Reptiles	No association
C. galli	Pavlásek, 1999	8.3 x 6.3	Chicken	No association
C. andersoni	Lindsay, 2000	7.4 x 5.5	Cattle	Rarely associated; individual reports from UK, Australia, and Malawi.
C. canis	Fayer, 2001	5.0 x 4.7	Dog	Epidemiologically linked to diarrhea in children in Peru; occasional sporadic cases in various, especially non-industrialized countries; some cases report contact with dogs.
C. hominis	Morgan-Ryan, 2002	4.9 x 5.2	Humans	Common cause of diarrheal disease in sporadic cases and outbreaks.
C. molnari	Sijta-Bobadilla, 2002	4.7 x 4.5	Fish	No association
C. suis	Ryan, 2004	4.6 x 4.2	Pig	Rarely associated; individual reports from UK and Peru involving contact with pig
C. scophthalmi	Alvarez Pellitero, 2004	4.4 x 3.9	Fish	No association
C. fayeri	Ryan, 2008	4.9 x 4.3	Marsupials	Rarely associated; one case reported in Australia of a person in contact with marsupials
C. ryanae	Fayer, 2008	3.7 x 3.2	Cattle	No association
C. fragile	Jirku, 2008	6.2 x 5.5	Black spined toad	No association
C. macropodum	Power, 2008	5.4 x 4.9	Eastern grey kangaroo	No association
C. xiaoi	Fayer, 2009	3.9 x 3.4	Sheep	No association

C. ubiquitum	Fayer, 2010	5.0 x 4.7	Mammals	Sporadic cases in various countries possibly involving untreated water supplies contaminated by animals hosts
C. tyzzeri	Zhao, 2012 Invalid by Slapeta et al, 2012	4.6 x 4.2	Mice	Rarely associated, individual reports from Czech Republic involving contact with wild mice
C. viatorum	Elwin, 2012	5.4 x 4.7	Humans	Sporadic cases in UK and Sweden
C. scrofarum	Kvac, 2013	5.2 x 4.8	Pig	Rarely associated, reports from Czech Republic involving contact with pigs

Data compiled from Slapeta, 2012 and Chalmers and Katzer 2013

1.4. Genomics of *Cryptosporidium* species

The era of *Cryptosporidium* genomic research began in the late nineties. The National Institute of Allergy and Infectious Diseases funded a consortium of three universities (University of Minnesota, Virginia Commonwealth University and Tufts University) to sequence the genome of two species of *Cryptosporidium*: *C. parvum* and *C. hominis* species responsible for the majority of human infections. The primary motivation for subsequent sequencing efforts was the public health importance of *C. parvum* and *C. hominis*, the lack of effective drugs to treat cryptosporidiosis, and the lack of research tools (Woods, Nesterenko, and Upton, 1996). The genome sequencing became important in order to address a wide range of questions, from basic biology to the identification of drug targets, from taxonomy to population biology (Striepen et al., 2004).

The widely distributed *C. parvum* IOWA isolate and *C. hominis* TU502 isolate from a Ugandan child were chosen for sequencing. The genome sequences of *C. parvum* and *C. hominis* isolates, each comprising eight chromosomes, showed similar genome sizes of 9.11 and 9.16 Mb, respectively, with 95-97% DNA sequence identity and ~30% GC content, with no large indels (insertion or deletion of bases in the DNA) or evident rearrangements when *C.*

hominis contigs were mapped to the *C. parvum* genome (Abrahamsen et al., 2004; Xu et al., 2004). Their sets of protein and RNA coding genes are essentially identical; thus phenotypic differences between them such as host range are presumed to be due to subtle polymorphisms (e.g., in micro- and mini-satellite repeat lengths (Tanriverdi and Widmer, 2006)) and differential gene regulations (Xu et al., 2004). Comparing *Cryptosporidium* genome to other eukaryotes, including the apicomplexans, a streamlining is achieved by a reduction in gene number, number of introns, and intergenic lengths. In addition to this, the genome sequencing and assembly of *C. muris* strain RN66 (unpublished but deposited into public databases) (http://CryptoDB.org/) are essentially complete.

Cryptosporidium genome analysis has revealed extremely streamlined metabolic pathways and a lack of many cellular structures and particular metabolic pathways found in general in eukaryotes or more specifically in *Apicomplexa* (Wanyiri and Ward, 2006). Energy metabolism is largely dependent from glycolysis, and both aerobic and anaerobic metabolisms are available, thus conferring environmental flexibility (Barta and Thompson, 2006). Limited biosynthetic capabilities and minimal metabolism have been reported, suggesting a large dependence on nutrient acquisition from the host (Rider and Zhu, 2010). Cryptosporidians have completely lost their apicoplast, a non-photosynthetic plastid, though few of its proteomic relicts can be identified in the nuclear genome by their homology to algal and cyanobacterial genes. *C. parvum* and *C. hominis* have a degenerate 'mitosome' instead of a mitochondrion, and have lost the mitochondrial genome and nuclear genes for many mitochondrial proteins, including those required for the Krebs cycle, oxidative phosphorylation, and fatty acid oxidation (LaGier et al., 2003). However, the existence of a relict mitochondrion was subsequently confirmed by ultra-structural studies (Keithly et al., 2005). Genes for de novo biosynthesis of amino acids, nucleotides, and sugars, as well as mechanisms for splicing RNA and gene silencing are also absent. The loss of these genes could mean that *C. parvum* and *C. hominis* rely heavily on scavenging nutrients from the host for energy production (Abrahamsen et al., 2004; Huang et al., 2004; Templeton et al., 2010; Toso and Omoto, 2007; Xu et al., 2004).

Nutrient uptake is accomplished by a lineage-specific expanded array of transporters of sugars, amino acids, and fatty acids (Abrahamsen et al., 2004; Xu et al., 2004). At least, 31 genes are very likely to have been acquired via intracellular transfer from organelle genomes or via horizontal transfer from bacteria (Huang et al., 2004), including a few that are not

conserved in other apicomplexans, e.g., genes encoding α-amylase, Inosine monophosphate dehydrogenase and thymidine kinase (Striepen et al., 2004).

A comprehensive genome database, CryptoDB (http://CryptoDB.org/), serves as an online public interface for *Cryptosporidium* genome sequences (Puiu et al., 2004). This website offers access to sophisticated tools which enable the identification of genes based on text, sequence similarity, and motif queries (Striepen and Kissinger, 2004). CryptoDB has recently been incorporated into a more comprehensive assembly of parasite databases under EuPathDB (Aurrecoechea et al., 2007). The genome sequences for *C. parvum*, *C. hominis* and *C. muris* have also been produced and provide a valuable comparison between them. An additional metabolic resource for *Cryptosporidium* which is also accessible through CryptoDB, is the CryptoCyc database (http://apicyc.apidb.org/C*PARVUM*/server.html) and is useful for comparison with predicted metabolism pathways in other organisms (Caspi et al., 2012).

The sequencing of *Cryptosporidium* genomes has revealed a vast amount of information, contributing to a better knowledge of microbial biology, pathogenicity, evolution and virulence. The quest for the molecular basis of virulence has exploited these genomic data to search for genes that may ultimately unravel the regulation of virulence, host range, and transmissibility at the genetic level (Casadevall and Pirofski, 2001).

Several comparative genomics studies have been performed since the completion of genome sequences from apicomplexan parasites of medical and veterinary importance.

Phylogenomics analyses of *C. parvum* genes provided a preliminary estimate of the number of loci that are likely to have originated through lateral gene transfer (Huang et al., 2004). Templeton and colleagues (Templeton et al., 2004) showed that *Cryptosporidium* spp. and *Plasmodium* spp. share over 150 ancestral “apicomplexan” proteins, involved mainly in interactions with eukaryotic host cells and in the biogenesis of the apical complex. Gordon and Sibley (Gordon and Sibley, 2005) used genome sequences of *Toxoplasma gondii*, *Plasmodium* spp., *Cryptosporidium* spp., and *Theileria* spp. to show the conservation of actin-like proteins among these parasites, which rely on actin-based motility for cell invasion, while comparative genomics of *Plasmodium* spp., *Cryptosporidium* spp., and *Toxoplasma gondii* revealed that calcium-regulated proteins (plant-like pathways for calcium release channels and calcium-dependent kinases) were also conserved (Nagamune and Sibley, 2006). In addition to conserved genes, comparative genomics can identify unique, novel, and

uncharacterized virulence genes. Kuo et al. compared the genome sequences of three apicomplexan parasites (*Plasmodium*, *Theileria*, and *Cryptosporidium*) and showed that as many as 45% of the *Cryptosporidium* genes could be considered genus specific (Kuo, Wares, and Kissinger, 2008). Although the predicted gene counts were not identical, the papers reporting the genomes of *C. parvum* (3,952 genes) and *C. hominis* (3,994 genes) found no evidence for unique genes between the species, noting that the variation that appeared to be present reflected primarily gaps in one genome or the other, and speculating that the phenotypic differences likely arose from polymorphisms in coding regions and from differences in gene regulation. However, the comparative genomics study by Kuo et al., identified 334 putative *C. hominis*-specific genes and 178 putative *C. parvum* specific genes by interrogations of the *Cryptosporidium* database into which the gene sequences had been placed (Kuo, Wares, and Kissinger, 2008). Furthermore, a study by Widmer and colleagues, which also aimed to investigate the genetic basis of *Cryptosporidium* host specificity, used genome-wide comparisons of *C. parvum* zoonotic, *C. parvum* anthroponotic, and *C. hominis* isolates. Those authors reported that for some genetic loci, there was actually more sequence similarity between *C. parvum* anthroponotic and *C. hominis* strains than there was between *C. parvum* anthroponotic and *C. parvum* zoonotic strains (Widmer et al., 2012).

In the post-genomic era, the discovery and validation of protozoan virulence factors in particular have been accelerated by the application of technological advances, including comparative genomics, transcriptomics, microarrays, and reverse genetics including gene replacement and small interfering RNA (siRNA). In conclusion, the era of cheap sequencing promises to advance our understanding of the evolution of *Cryptosporidium*.

2. Epidemiology

Cryptosporidiosis is a cosmopolitan infection reported on every continent except Antarctica, with the incidence and prevalence varying around the world. The global prevalence of human cryptosporidiosis is between 0.5 and 2% in industrialized countries and can exceed 10% in developing countries (Guyot, Sarfati, and Derouin, 2012).

Worldwide environmental and veterinary surveillance data reveal the presence of *Cryptosporidium* spp. in water treatment systems, which represents an unacceptable health risk, particularly in sensitive (elderly, pregnant women, children) and immunocompromised

populations (HIV-positive and transplanted patients). However, the greatest burden of cryptosporidiosis occurs in developing countries because of the disseminated status of malnutrition in children and the highest world prevalence of HIV infection in certain geographic areas of this region, especially in Africa (Fergusson and Tomkins, 2009). It is very difficult to quantify as estimates vary widely, even among studies in the same geographic regions, as a result of differences in study design, sample size, age range, immunity status, severity of the disease, and sensitivity of the diagnostic methods. Nevertheless, it is recognized in most epidemiological studies that the prevalence of cryptosporidiosis is probably underestimated because infection with this parasite can be asymptomatic and difficult to detect in immunocompetent people. Studies of children in daycare centers, suggest that asymptomatic excretion of oocysts is not uncommon. For instance, in Trujillo State, Venezuela, *Cryptosporidium* oocyst shedding was reported in 89% of healthy children attending day-care centers (Miller et al., 2003). Recent publications agree on the fact that the incidence of cryptosporidiosis is significantly increasing worldwide (Chalmers et al., 2011b; Fournet et al., 2013; Yoder, Harral, and Beach, 2010; Yoder et al., 2012).

The epidemiology of cryptosporidiosis is influenced by difference factors: the infective dose is low (one to ten oocysts); (ii) oocysts are immediately infectious when excreted in the environment; (iii) oocysts are stable and highly resistant to chlorine disinfection and survive for months in the environment; and (iv) environmental dispersal can lead to the contamination of drinking water and food (Caccio et al., 2005).

Cryptosporidium oocysts are most commonly transmitted by the fecal-oral route through direct host to host contact, and indirect contamination of water or food. Direct and indirect transmission between infected hosts and susceptible individuals is favored by high population densities and by close contact (Caccio et al., 2005). Cases of human-to-human transmission have been reported between family members, sexual partners, children, and in hospitals. Zoonotic transmission has been confirmed by epidemiological studies concerning farm and companion animals, and veterinary workers. Food handled by a contaminated or infected person, and food that has been exposed to contaminated water have been sources for food-borne cryptosporidiosis. Food grown in soil fertilized with manure could also be considered a potential source of infection. However, contaminated water represents the major source of infections for humans (Ramirez, Ward, and Sreevatsan, 2004).

During a period of almost hundred years, more than 300 waterborne protozoan parasitic outbreaks have been reported worldwide being *Cryptosporidium* spp. the main etiological agent (present in 60.3% of waterborne outbreaks) (Karanis, Kourenti, and Smith, 2007) followed by other parasites such as *Giardia* spp. and *Toxoplasma gondii* (Baldursson and Karanis, 2011). In particular, more than 160 waterborne outbreaks of cryptosporidiosis have been reported in the last decade implicating contaminated drinking and recreational water (Yoder, Harral, and Beach, 2010; Yoder et al., 2012).

C. hominis and *C. parvum* are known as the major agents causing human cryptosporidiosis both in immunocompetent and in immunocompromised individuals and their prevalence varies in different regions of the world. Epidemiological studies showed that *C. hominis* is more prevalent in Australia, West Europe, India, and Africa (Fournet et al., 2013; Helmy et al., 2013; O'Brien, McInnes, and Ryan, 2008; Sharma et al., 2013), whereas *C. parvum* human cases are more prevalent in Chile, Turkey, Middle East and Thailand (Alyousefi et al., 2013; Iqbal, Khalid, and Hira, 2011; Neira et al., 2012; Nuchjangreed et al., 2008; Taghipour et al., 2011; Usluca and Aksoy, 2011). Additionally, *C. meleagridis* has been confirmed as an emerging human pathogen, being responsible for 10% of cases in Peru, where its prevalence is as high as the prevalence of *C. parvum* (Cama et al., 2008). Geographic variations in the distribution of *C. parvum* and *C. hominis* can also occur within a country. For example, *C. parvum* is more common than *C. hominis* in rural states of Ireland (Zintl et al., 2009).

Molecular tools have provided new insights into *Cryptosporidium* taxonomy helping to understand its epidemiology. Subtyping tools have been used extensively in studies of the transmission of *C. hominis* in humans, and *C. parvum* in humans and ruminants. The DNA sequence analysis of the 60 kDa glycoprotein (gene) is one of the most employed subtyping tools. The gene has tandem repeats of the serine-coding trinucleotide TCA, TCG or TCT at the 5’ end of the gene. In addition to variations in the number of trinucleotide repeats, there are extensive sequence differences in the non-repeat regions, which categorize *C. parvum* and *C. hominis* into several subtype families (Xiao, 2010).

Nowadays it is known that most of the outbreaks around the world were caused by the anthroponotic species *C. hominis* and an intra-specific characterization using this marker allowed the identification of the *C. hominis* subtype family Ib in most of the disease cases. The subtype Ib had very different subtypes from which the investigations have identified the

subtypes IbA9G3 and IbA10G2 commonly found in the majority of areas in the world (Xiao, 2010).

The IbA9G3 is usually observed in humans in Kenya, India, and Australia, and IbA10G2 is commonly seen in South Africa, Peru, USA, Canada, Australia, and European countries, as France, UK, Portugal, Spain and Ireland (Alves et al., 2006a; Cama et al., 2008; Chalmers et al., 2005; Fournet et al., 2013; Gatei et al., 2006b; Leav et al., 2002). IbA10G2 is responsible for more than half of the waterborne outbreaks in USA, and Canada (Boehmer et al., 2009). Most notably, *C. hominis* IbA10G2 subtype was the causal agent associated to the 1993 Milwaukee waterborne cryptosporidiosis outbreak (Corso et al., 2003). Other subtypes of *C. hominis* exist such as IbA13G3 subtype, which was seen in Peru (Cama et al., 2007), and several otherwise rare subtypes, IbA6G3, IbA19G2, IbA20G2, IbA21G2 and IbA23G2, found in Jordan and China (Feng et al., 2009; Hijjawi et al., 2010). Likewise, in most developing countries, humans with the subtype family Ie are mostly infected with IeA11G3T3, excepting Jamaica and China where IeA12G3T3 is seen (Alyousefi et al., 2013; Feng et al., 2009; Gatei et al., 2008; Sulaiman et al., 2005). As well, other rare subtypes such as IdA14 (Sulaiman et al., 2005), IdA19 (Trotz-Williams et al., 2006), Id A20 (Nazemalhosseini-Mojarad et al., 2011), IdA21 and IdA24 (Hijjawi et al., 2010) exist. IfA22G1 subtype is also described in Iran (Nazemalhosseini-Mojarad et al., 2011; Taghipour et al., 2011). Much less genetic diversity of *C. hominis* is observed in industrialized nations. In European countries and Australia, most *C. hominis* infections are caused by the Ib subtype family (Alves et al., 2006b; O'Brien, McInnes, and Ryan, 2008; Waldron, Ferrari, and Power, 2009; Wielinga et al., 2008; Xiao, 2010; Zintl et al., 2009). The majority of these cases had the IbA10G2 subtype and less commonly the IbA9G3. In addition, several unusual Ib subtypes (IbA18G1, IbA5G2T3 and IbA9G2T1) were also occasionally found (Jex et al., 2008; Jex et al., 2007; O'Brien, McInnes, and Ryan, 2008).

On the other hand, the *C. parvum* IIa subtype family is responsible of the majority of cryptosporidiosis outbreaks due to this specie. Particularly, the subtypes IIaA15G2R1, IIaA16G1R1, IIaA17G2R1, and IIaA19G2R1 are responsible for zoonotic cryptosporidiosis and have been found in both humans and ruminants. These subtypes are described in many geographic areas of the world, especially the subtype IIaA15G2R1 which is known as the major subtype of *C. parvum* in the world. It dominates in most of the countries around the world such as in USA, Canada, UK, Germany, Netherlands, Spain, Portugal, Slovenia, India, Japan and Yemen. Whereas other subtypes dominates in other countries such as IIdA20G1 in

Egypt, Jordan, Kuwait and Iran, IIaA18G3R1 in Australia and North Ireland, IIaA15G1R1 in Lebanon (Osman et al, data not published), and IIaA16G1R1 in Serbia (Xiao, 2010).

However, other subtype family, IIc described only in humans is also responsible of transmission of cryptosporidiosis mainly in developing countries (Wheeler et al., 2007; Xiao and Feng, 2008).

Seasonality is also associated to variations in incidence of cryptosporidiosis around the world. Usually in developed countries, incidence peaks during the summer when persons swim more often (Naumova et al., 2005; Yoder, Harral, and Beach, 2010; Yoder et al., 2012). The parasite is particularly suited to waterborne transmission as the environmentally resistant oocysts are shed in large numbers in feces (10^8–10^9 oocysts/gram), have a low infectious dose and are highly resistant to chlorine disinfection (Yoder, Harral, and Beach, 2010). Swimming in untreated recreational waters, such as lakes and dams, is particularly hazardous to public health as there are additional potential sources of contamination (Karanis, Kourenti, and Smith, 2007). In particular, freshwater swimming was found to be a less risk factor compared to swimming in pools and water parks. High bather densities with routine use of recreational waters by incontinent persons, including diapered children and toddlers facilitate infections and outbreaks in public pools and water parks (Loganthan et al., 2012). In contrast, in developing countries, the prevalence of cryptosporidiosis can be higher during the rainy season because of heavy rains and runoff that can probably contaminate water sources especially in tropical countries (Abd El Kader et al., 2012; Iqbal, Khalid, and Hira, 2011). In the U.K. peaks of incidence during the spring and in the late autumn are associated with the farming activity and a peak in rainfall (Casemore, Wright, and Coop, 1997). In addition, cryptosporidiosis has been recognized too as an important cause of travelers' diarrhea (Paschke et al., 2011).

3. Clinical features

Clinical manifestations and the severity of cryptosporidiosis can vary from one person to another, especially depending on the immune status of the host.

In immunocompetent persons most cases are detected in children under 5 years of age (Chalmers and Davies, 2010). The incubation period has been reported from outbreaks as mean of 7 days from exposure (Chalmers, 2003). The most notable symptom in

immunocompetent individuals is watery and voluminous diarrhea, usually between 3 and 6 stools each day but sometimes can be even more (Chalmers and Davies, 2010). Mucus can be present in stools but blood and leukocytes are rare. Other symptoms such as abdominal cramps, anorexia, nausea, vomiting, weight loss, low grade fever and fatigue have also been reported (Fayer, 2004). In immunocompetent individuals, the duration of the symptoms is as mean 9 days and these clinical manifestations are self-limiting (Ripert and Guyot, 2003). Oocysts can be shed for a mean period of 7 days after disappearing of symptoms.

On the other hand, in immunodeficient patients, a depletion of CD4 + T cells is associated with a greater risk for developing cryptosporidiosis (Marcos and Gotuzzo, 2013). Immunocompromised populations at risk for diarrhea associated with *Cryptosporidium* infection include: HIV/AIDS patients, solid organ and hematopoietic stem cell transplant patients, persons with hypogammaglobulinemia, severe combined immunodeficiency, X-linked hyper-IgM syndrome, or people receiving anticancer therapy or immunosuppressive drugs (Chalmers and Davies, 2010). Particularly, some reports have described different presentations of cryptosporidiosis in patients with AIDS: 1. Asymptomatic infections, 2. Transient infections common in less strongly immunosuppressed patients, 3. Fulminant disease characterized by the passage of more than 2 liters of stools/day and found in those patients with a CD4 count less than 50/mm3; and 4. Chronic disease (Hunter and Nichols, 2002).

Some studies have reported health sequelae of cryptosporidiosis in immunocompetent patients (Hunter et al., 2004). Recurrence of gastrointestinal symptoms has been frequently reported, but also long term non gastrointestinal health effects of cryptosporidiosis have been described. A seronegative reactive arthritis, has been reported in adults (Hay, Winfield, and McKendrick, 1987; Ozgul et al., 1999) and children (Cron and Sherry, 1995; Shepherd, Smail, and Sinha, 1989) including one report of Reiter's syndrome (arthritis, conjunctivitis and urethritis) (Cron and Sherry, 1995). Reported joint pain was of longer duration in cryptosporidiosis cases than in healthy controls in a UK study, conducted 2 months after initial *Cryptosporidium* diagnosis (Hunter et al., 2004). That study also identified that joint pain, eye pains, recurrent headache, dizzy spells, and fatigue were significantly more common in *C. hominis* than in *C. parvum* cases.

In immunocompromised patients, cryptosporidiosis infection can become chronic and life-threatening because of frequent, watery evacuations leading to a very severe dehydration. In

some cases, extra-intestinal and extra-abdominal spread of infection had been reported (Fayer, 2004). Infection of gall bladder and bile ducts can result in cholangiopathy and sclerosing colangitis characterized by symptoms such as fever, non-radiating upper quadrant pain, nausea, vomiting, diarrhea and jaundice. Elevated serum bilirubin and liver enzymes can be associated. Symptoms of respiratory cryptosporidiosis can include cough, croup, wheezing and dyspnea (Arrowood, 1997).

3.1. Clinical manifestations associated to infecting *Cryptosporidium* species

The species most commonly found infecting humans are *C. parvum* and *C. hominis* (Table 2). Other species such as *C. meleagridis*, *C. felis*, *C. andersoni*, *C. canis* and *C. muris* have also been found in humans (Table 2), both immunocompromised and immunocompetent (Xiao and Ryan, 2004). Cases of multiple infections by two or three species of *Cryptosporidium* have been described in HIV + patients (Cama et al., 2006).

Although the clinical presentations of *C. parvum* and *C. hominis* infections are very similar, several variants have been reported. In a case control study conducted in England, it was found that *C. hominis* is more often associated with the occurrence of non gastro-intestinal symptoms, than *C. parvum*. These symptoms include ocular and articular pain, headache, and fatigue (Hunter et al., 2004). Some authors also reported a higher number of oocysts of *C. hominis* excreted in the feces compared to *C. parvum* (Xiao et al., 2001a).

In a cross-sectional study performed in Peru among HIV /AIDS patients, different species, genotypes and subtypes of *Cryptosporidium* were associated with different clinical manifestations (Cama et al., 2008). On one hand, infections with *C. hominis* subtype Id, *C. parvum*, *C. canis* or *C. felis* were associated with more severe disease characterized by chronic diarrhea and wasting syndrome in HIV/AIDS patients. This study also showed that infections with *C. meleagridis* were not associated with any clinical manifestation and these persons excreted fewer oocysts than those infected with other species (Cama et al., 2008). In patients infected with *C. hominis* subtype Id risk of diarrhea was higher, while it was lower in subjects infected with subtype Ib. Finally, subtype Ia was not associated with diarrhea (Cama et al., 2008).

A series of studies in healthy adult volunteers to test the phenotypic characteristics of different species and isolates of *Cryptosporidium* were performed (Chappell, Okhuysen, and White, 2003). In these experimental studies, after oral infection with *C. parvum*, the onset of

diarrhea was observed between 4 and 7 days post-infection. The oocyst excretion usually started between 6 and 8 days after the challenge, lasting from 3 to 8 days. These studies showed that different strains of *Cryptosporidium* (UCP, Iowa, TAMU) had different ID50s (infectious dose 50%), indicating differences in their infectivity. It was also found that people with pre-existing antibodies against *Cryptosporidium* showed relative resistance to re-infection with the Iowa strain (Chappell, Okhuysen, and White, 2003). *C. hominis* (TU502) was also tested and it was found that this species could cause infection and disease in healthy adults as *C. parvum* (Chappell et al., 2006).

3.2. *Cryptosporidium* and cancer (some clinical evidences)

There were some speculations about possible associations between *Cryptosporidium* infection and neoplasia in humans (Benamrouz et al., 2012a). The association of cryptosporidiosis and colonic adenocarcinoma was suspected in the case of a Spanish patient carrying both, who died rapidly after the onset of symptoms (Izquierdo et al., 1988). A cryptosporidiosis case of the biliary tract clinically mimicking a pancreatic cancer in an AIDS patient was also reported (de Souza Ldo et al., 2004). Furthermore, an association between X linked immunodeficiency with hyper IgM syndrome, and carcinoma of the liver, pancreas and biliary tree was described in children by Hayward *et al*. (Hayward et al., 1997). These authors proposed that the mutation responsible for this defect may favor the colonization of the biliary epithelium by different pathogens, including *Cryptosporidium*. A chronic infection and inflammation by this parasite will follow, perhaps being the inflammation the cause of the malignant transformation (Hayward et al., 1997).

More recently, epidemiological studies in Poland reported a frequency of 18% and 12.6% of cryptosporidiosis in patients with colorectal cancer of recent diagnosis before any immunosuppressive treatment (Sulzyc-Bielicka et al., 2012) being these prevalences higher compared with the burden of cryptosporidiosis for the general European population (Semenza and Nichols, 2007).

Finally, Shebl et al (2012) analyzed 320 colorectal cases occurred among 471 909 persons with AIDS and cryptosporidiosis. Colon squamous cell carcinoma risk was significantly elevated among AIDS patients who had cryptosporidiosis at the same time (Shebl, Engels, and Goedert, 2012).

According to Kutikhin et al (2013), *Cryptosporidium* should be included in the extended list of the agents that can cause cancer development based on basic and epidemiological evidences (Kutikhin, Yuzhalin, and Brusina, 2012).

4. Pathogenesis and Immunity

The high infectious power of *Cryptosporidium* isolates from human or animal origin has already been reported (Chappell et al., 1996). Some studies have been conducted so as to determine the infectivity of this parasite by looking for the minimal oocyst infective dose. Experimental animal models showed that a single oocyst of *C. parvum* is able to infect immunosupressed SCID and C57BL/6 adult mice (Benamrouz et al., 2012b; Yang et al., 2000). Similarly, for *C. meleagridis* a single oocyst was able to infect immunosupressed C57BL/6 adult animals (Huang et al., 2003), In parallel, volunteer studies provided evidences showing that low challenge of *Cryptosporidium* oocysts was infective to healthy humans (Chappell et al., 1996; DuPont et al., 1995). Experiments showed an estimated ID50 of 1042, 87 and 9 when persons were challenged with *C. parvum* UCP, Iowa and TAMU isolates respectively, and an ID50 of 10 when the challenge was with *C. hominis* (TU 502) isolate (Chappell et al., 2006). Moreover, a mathematical model based on epidemiological data from the Milwaukee outbreak suggested that some individuals developed cryptosporidiosis following the ingestion of only one oocyst (Haas and Rose, 1994). All these observations indicate that low inocula are able to induce infection in both animals and humans. However, the susceptibility of animals and humans to *Cryptosporidium* infection depends on many factors, notably the intrinsic diversity of the isolates, and also the host immune response (Okhuysen and Chappell, 2002; Petri et al., 2008).

Particularly, over 25 putative *Cryptosporidium* virulence factors have been described (Table 1) (Bouzid et al., 2013). These virulence factors are considered as the processes and substances by which the parasite initiates and maintains the infection in the host (Fayer, Orlandi, and Perdue, 2009) They are involved in adhesion, colonization, invasion, and host immune evasion and they can participate at any time during the life cycle (Table 1) (Fayer, Orlandi, and Perdue, 2009).

4.1. Adherence to and invasion of epithelial host cells

The initial host-parasite interaction of attachment and invasion are critical primary events for the pathogenesis. These processes are facilitated by several proteins secreted and successively exocytosed by the organelles of the apical complex (micronemes, rhoptries and dense granules) (Tzipori and Ward, 2002). Different molecules facilitating the initial steps in the establishment of infection are described below:

The circumsporozoite-like antigen (CSL) is a soluble glycoprotein of 1300-kDa, located in apical complex of sporozoïtes and merozoïtes (Langer, Schaefer, and Riggs, 2001; Riggs et al., 1997). It contains a ligand involved in the process of attachment and invasion that binds specifically to a receptor on microvillar surface of human and calf intestinal epithelial cells (Boulter-Bitzer, Lee, and Trevors, 2007; Bouzid et al., 2013). Indeed, CSL-reactive monoclonal antibody (MAb 3E2) prevents sporozoite attachment and invasion in vitro and passively protects against the infection in vivo (Riggs et al., 1997).

Glycoprotein 900 (gp 900) is a large mucin-like glycoprotein of *C. parvum* located in micronemes and at the surface of invasive merozoites and sporozoites. This protein participates in gliding motility and mediates host epithelial cell invasion (Barnes et al., 1998; Deng, Rutherford, and Abrahamsen, 2004; Petersen et al., 1992).

Sporozoite and merozoite cell surface protein gp 15/40/60 complex has been described (Cpgp40/15 in *C. parvum* and the Chgp40/15 in *C. hominis*). gp40 and gp15 glycoprotein are considered as proteolytic products of the larger molecule (Okhuysen and Chappell, 2002) under the action of subtilisin-like serine protease activity (Wanyiri et al., 2009). The analysis of polymorphic regions of sequence is used to classify *Cryptosporidium* isolates into subtypes (Bouzid et al., 2013). Interestingly, some studies report differences in severity of clinical manifestations between species and strains of *Cryptosporidium* but such association is not always found (Widmer and Sullivan, 2012). gp40, mucin-like glycoprotein is localized at the apical complex of the invasive stages of the parasite and is shed from its surface while gp15 is on the surface of the sprozoite and is shed in trails during gliding movements (Boulter-Bitzer, Lee, and Trevors, 2007; Bouzid et al., 2013; Cevallos et al., 2000; Deng, Rutherford, and Abrahamsen, 2004). gp40-specific antibodies and lectins neutralize infection of intestinal epithelial cells (Caco-2 cells) in vitro (Cevallos et al., 2000; Lievin-Le Moal, 2013). In addition, both gp40 and gp15 display O-linked-α-N-acetyl-galactosamine (α-GalNAc) determinant, which is thought to be involved also in host cell attachment and invasion since

lectins that recognized these determinants block sporozoite attachment (Boulter-Bitzer, Lee, and Trevors, 2007; Bouzid et al., 2013).

Furthermore, a Gal/GalNAc –specific lectin, p30, has been identified and detected in *C. parvum* and *C. hominis* complexes with both gp900 and gp40. This complex prevents sporozoite attachment to the cultured intestinal epithelial cells, suggesting its role in mediating attachment to and invasion of epithelia (O'Hara and Chen, 2011).

Thanks to the complete genome sequencing of both *C. parvum* and *C. hominis*, a single locus of seven small mucin sequences (CpMUC1 to -7) was identified (O'Connor et al., 2009). After investigation of their role using specific antibodies against CpMuc4, it was concluded that this proteins has also a role in the phases of attachment and invasion (O'Connor et al., 2009; O'Hara and Chen, 2011).

Sporozoite surface protein p23, identified as an antigen with neutralization-sensitive epitope (Boulter-Bitzer, Lee, and Trevors, 2007), is also predicted to contain mucin-type O-glycosylation sites (O'Connor et al., 2009). Enriquez and Riggs reported that the use of dimeric anti-p23 IgA MAbs significantly ($p<0,005$) reduces *C. parvum* infection in neonatal mice (Enriquez and Riggs, 1998).

Thrombospondin-related adhesive protein *Cryptosporidium*-1 (TRAP-C1) is a microneme protein of 76 k-Da localized on the apical pole of sporozoites (Boulter-Bitzer, Lee, and Trevors, 2007; Bouzid et al., 2013; O'Hara and Chen, 2011). Studies have described the involvement of TRAP and TRAP related protein in gliding motility and invasion (O'Hara and Chen, 2011). TRAP proteins are characterized by their ability to link receptors expressed on the host cells to the parasite acto-myosin motor (Boulter-Bitzer, Lee, and Trevors, 2007).

A *C. parvum* subtilisin-like serine protease (CpSUB) has also been characterized (Wanyiri et al., 2009) . They suggest that this protein may be a likely candidate for the protease activity in *C. parvum* as well as for the processing of gp40/15. These studies indicate also that CpSUB1 plays a significant role in infection of host cells.

Phospholipases, proteases and haemolysins are potentially involved in the cellular damage (Okhuysen and Chappell, 2002). Haemolysin H4 is an haemolytic peptide (Steele et al., 1995)which has sequence similarity with the haemolysin of enterohemorrhagic (EHEC) *Escherichia coli* 0157:H7. Its function is yet unknown. However, its ability to disrupt membranes suggests roles in cellular invasion, or disruption of vacuolar membranes.

Proteases have several functions such as degradation of nutrient proteins, invasion of host tissues, and evasion of host immunity (Bouzid et al., 2013). Three proteases activity have been identified in *Cryptosporidium* sporozoite: a cysteine, a serine endopeptidase and an aminopeptidase (Okhuysen and Chappell, 2002). The two later are probably involved in exystation process (Forney, Yang, and Healey, 1997; Okhuysen et al., 1996). It has been also suggested that these proteases are important in the initial stage of *Cryptosporidium* infection(Okhuysen and Chappell, 2002).

Heat shock proteins (HSPs) are a large conserved family of polypeptides. In *Cryptosporidium*, two HSPs have been described and sequenced, HSP90 and HSP70 (Khramtsov et al., 1997; Woods et al., 1999). It has been demonstrated for *Toxoplasma gondii*, that quantitative and qualitative differences in the expression of HSP are directly related to parasite virulence(Okhuysen and Chappell, 2002). However for *Cryptosporidium* further investigations are necessary before establishing such association between HSP protein and virulence.

In addition, a *C. parvum* ATP-binding cassette (ABC) transporter gene which could be involved in the nutrition of the parasite has been characterized (Perkins et al., 1999). This transporter gene share structural similarities with bacterial genes, which are critical in producing secretory diarrhea (Bouzid et al., 2013).

The proteins described above are assumed to be involved in mediating the attachment and invasion processes as well as being involved in the pathogenesis process of *Cryptosporidium* infection. However, establishing the real functional role of each putative virulent factor is still complicated by the fact that we are still unable to propagate and genetically manipulate the parasite.

4.2. Epithelial cellular processes initiated by *Cryptosporidium* infection

Cryptosporidium infection results in several cellular damages like the alteration of the cytoskeleton with the–disruption of tight cell junctions, a loss of barrier function and the modulation of apoptosis (Adams et al., 1994; Okhuysen and Chappell, 2002).

Cytoskeleton remodeling

Cryptosporidium infection induces cytoskeletal changes that modulate a localized actin reorganization and channel/transporter insertion (O'Hara and Chen, 2011). Several studies outline that infection of intestinal and biliary epithelial cells requires host cell actin polymerisation and cytoskeleton remodeling (Chen and LaRusso, 2000). This polymerization uses the actin branching and nucleation machinery of the Arp2/3 complex of proteins (Elliott et al., 2001). Multiple signaling axes have been identified to modulate actin reorganization and *Cryptosporidium* internalization such as PI3-kinase and the guanine exchange factor, Frabin-dependent activation of the small GTPase, CDC42 and c-Src -dependent activation of cortactin (O'Hara and Chen, 2011).

Modulation of apoptosis

Several studies report the ability of intracellular protozoan parasite to inhibit apoptotic programme of the host cell such as *Toxoplasma gondi*, *Trypanosoma cruzi*, *Leishmania* sp. or *Theileria* sp. (Heussler, Kuenzi, and Rottenberg, 2001).

It has been demonstrated that *C. parvum* activates nuclear factor κB (NF κB) preventing epithelial cell apoptosis in biliary epithelial cells (Chen et al., 2001). Indeed, NF κB is involved in the activation of numerous intracellular survival signals including C-myc oncogene (Chen et al., 2001). However, several studies highlight that *C. parvum* not only inhibits cell apoptosis but is also able to modulate this process, inhibiting apoptosis at the trophozoïte stage and promoting this process at the sporozoïte and merozoïte stage (Mele et al., 2004). Additionally, the analysis of gene transcript of host cells reveals that early in infection, genes with antiapoptotic roles are up-regulated and genes with apoptotic roles are down-regulated. Later in infection, proapoptotic genes are induced and antiapoptotic genes are then down-regulated, suggesting a biphasic regulation of apoptosis (Liu et al., 2009).

Immune responses to Cryptosporidium infection

In immunocompetent individuals, the disease induced by *Cryptosporidium* infection is self-limiting. On the contrary, for immunocompromised patients with an impaired immune system (as AIDS patients), the infection can become chronic and life-threatening. The immune response to this parasite is poorly understood. However, both innate and adaptative immune systems seem to play a key role. Several components involved in this response have been

summarized in different reviews (Laurent et al., 1999; Leitch and He, 1999; O'Hara and Chen, 2011; Petry, Jakobi, and Tessema, 2010; Riggs, 2002; Zu et al., 1992).

Innate immunity

The first barrier against invading microbes is the intestinal epithelial cells (IECs) (Magalhaes, Tattoli, and Girardin, 2007). These cells expressed Toll-like receptors (TLRs) and intracellular Nod-like receptors (NLRs) that permit microbial recognition which results in the expression of proinflammatory cytokines such as IL-18 (IFN-γ inducing factor) (Okazawa et al., 2004) and chemokines from C, C-C and C-X-C classes (Petry, Jakobi, and Tessema, 2010). IECs contribute also to the antigen processing and presentation via the major histocompatibility complex (MHC) (Hershberg and Mayer, 2000).

Another significant player in innate response against *C. parvum* infection is IFN-γ. Indeed, it has been demonstrated that adult BALB\c mice treated with neutralizing anti- IFN-γ greatly enhanced the oocyst shedding (Ungar et al., 1991).

In addition, free radical nitric oxide (NO) (produced by macrophage and neutrophils) synthesis has been reported to be significantly increased in *C. parvum* infected mice and piglet while the administration of antioxidant has shown to exacerbate *C. parvum* infection (Gookin et al., 2006; Leitch and He, 1999). Thus, NO appeared to play a protective role (Petry, Jakobi, and Tessema, 2010).

Other immune cells are assumed to be involved in host resistance such as macrophage and neutrophils (Takeuchi et al., 2008) and dendritic cells (involving IFNα/β) (Hayward et al., 2001).

Recently, the role of the complement in immunity to *C. parvum* which is activated by classical and lectin pathways leading to the deposition of C3b on the parasite was reported (Petry et al., 2008).

Adaptive immunity

The importance of T cells in the immune response against *Cryptosporidium* infection have been demonstrated in several studies using different models like congenitally athymic mice T cell deficient (nude) mice (Heine, Moon, and Woodmansee, 1984), SCID mice immunologically reconstituted with Human or murine lymphocytes (Mead et al., 1991) and

BALB/c depleted from T cells (McDonald et al., 1992). It, thus, highlights the importance of T-lymphocytes for the recovery from *Crypotsporidium* infection.

Moreover, CD4+ helper T-cells has been determined to be crucial for the resolution of established *C. parvum* infection in SCID mice (Chen, Harp, and Harmsen, 1993). In addition, MHC-II (important for antigen-presentation to CD4+ T-cells) deficient mice have been reported to be more susceptible to *C. parvum* infection than MHC-I (important for the function of CD8+ T-cells) deficient mice. Nevertheless, other studies on calves (Abrahamsen et al., 1997) and BALB/c mice (McDonald et al., 1994) showed that CD8+ is also important during host immune response.

It has been reported that in addition to the involvement of IFN-γ during the innate immune response to the parasite, the cytokine is also a key player during adaptive immune response (Petry, Jakobi, and Tessema, 2010). In contrast to Th1 response, the role of Th2 response is more unclear. Several experiments based on depletion of IL4, lead Mc Donald et al to conclude that "the most effective Th response to control cryptosporidial infection may be a dynamic one in which there is a strong early Th1 response but the later maturation of a more balanced response with a Th2 component may facilitate parasite removal" (McDonald, 2000).

Finally, although some works report the presence of parasite-specific serum IgG as well as a systemic and mucosal IgA responses in infected mice, the protective role of antibodies is still under discussion given that high titres of parasite-specific IgG/IgA and mucosal IgA can be found in AIDS patients with chronic cryptosporidiosis (Petry, Jakobi, and Tessema, 2010).

4.3. *Cryptosporidium* and cancer (some experimental evidences)

The ability of *C. parvum* to induce gastrointestinal neoplastic changes was established experimentally in severe combined immunodeficiency (SCID) mice treated or not with dexamethasone (Figure 3). These animals developed adenomas with low or high-grade intraepithelial, intramucosal or invasive neoplasia in different area of digestive tract (stomach, duodenum and ileocaecal region). These neoplastic lesions appeared as soon as 45 days post infection (PI) after infection with *C. parvum* IOWA strain, whatever the inculum size used (from one to 10^7 oocyst) (Benamrouz et al., 2012b; Certad et al., 2007). SCID mice, infected with *C. parvum* TUM1 (strain isolated from animal feces) or a *C. parvum* strain isolated from human stools, developed a fulminant cryptosporidiosis associated with invasive

gastrointestinal and biliary adenocarcinoma (Certad et al., 2012; Certad et al., 2010). Neoplastic lesions induced by the latter progressed through all layers of the digestive tract to the subserosa and spread via blood vessels (Certad et al., 2012). No data about the mechanism of *C. parvum*-induced neoplasia are available but it is well known that this parasite can interfere with signaling pathways of the host cell as stated above.

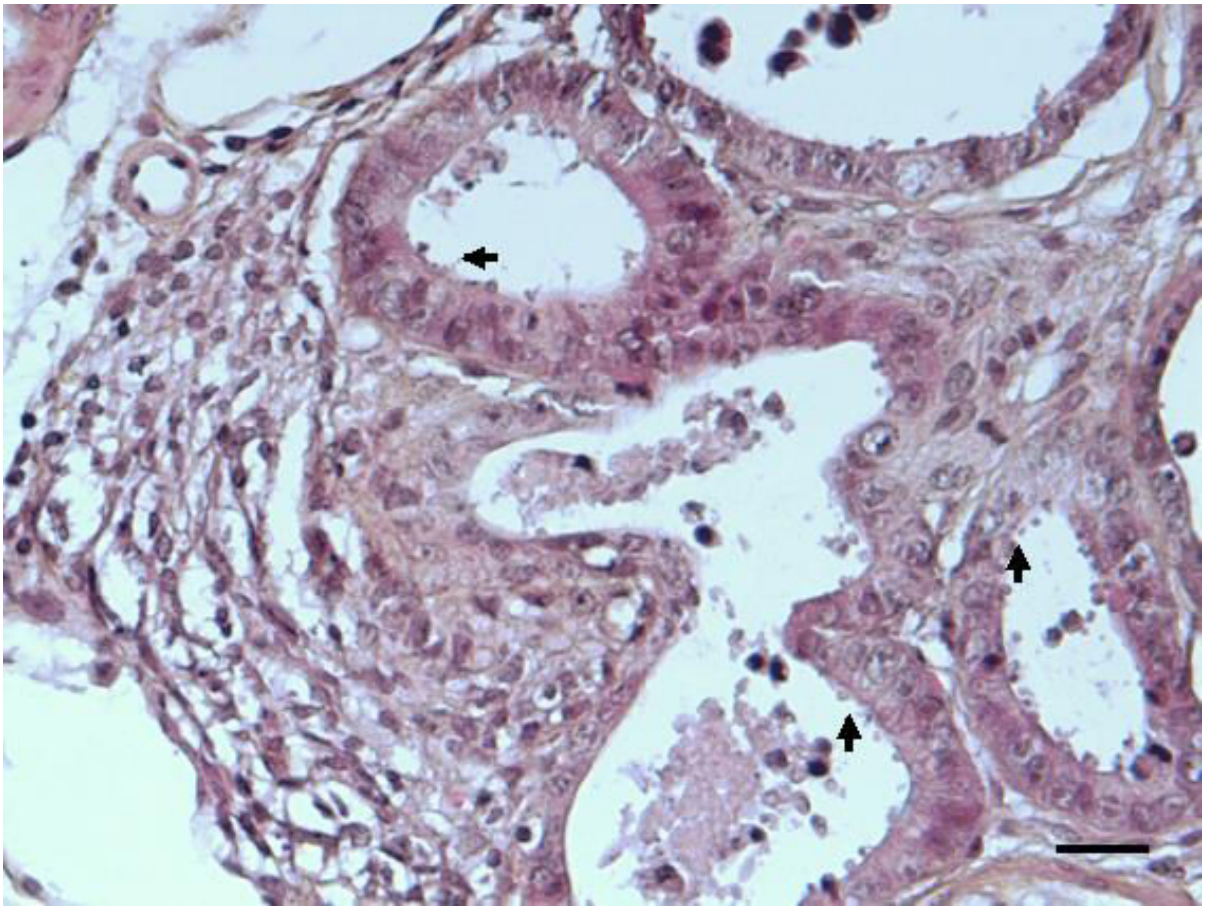


Figure 3. Ileo-caecal neoplastic lesions in a dexamethasone-treated SCID mice infected with *C. parvum*. Numerous parasites (arrows) inside the glands. Bar= 20 mm (hematoxylin and eosin). This photomicrograph was kindly provided by Dr. Colette Creusy from the "Service d'Anatomie et de Cytologie Pathologiques, Groupe Hospitalier de l'Université Catholique de Lille, France".

5. Diagnosis

The precise diagnosis of *Cryptosporidium* infection is essential for the control of this disease in humans and for the understanding of the complexities of its epidemiology. There are various techniques that can be used to detect *Cryptosporidium* in human and environmental samples. For the diagnosis of cryptosporidiosis microscopical examination is useful to look for the presence of oocysts in stools, duodenal biopsies, the biliary liquid or the sputum and bronchoalveolar lavage when a broncho-pulmonary location is suspected (location exceptional and normally associated with intestinal cryptosporidiosis) (Guyot, Sarfati, and Derouin, 2012).

However, oocysts are not easily identifiable during classical parasitological examination and must be searched after specific staining techniques. Therefore, the clinician should order a specific prescription (Guyot, Sarfati, and Derouin, 2012). The basic methods to detect oocysts are staining methods because they are fast, very specific and they have a low cost. In addition, haematoxylin and eosin can be used for the histological confirmation of the diagnosis when biopsies are available.

5.1. Staining methods

Various staining techniques have been employed to help in the detection of *Cryptosporidium* oocysts from other protists. The simplest, least expensive stain and most widely used is modified Ziehl–Neelsen (Henriksen and Pohlenz, 1981). This technique is based on the fact that oocysts can be stained with carbol-fuchsin retaining the dye in the decolorizing step. The acid fastness of the oocyts allows the differentiation from the stools that can be visualized by counter staining with malachite green (Figure 4D) (Henriksen and Pohlenz, 1981).

Besides acid-fast staining, other techniques have been described such as the Auramine O phenol staining (Figure 4C) . This method is a fluorescent stain that offers increased sensitivity but decreased specificity compared to other detection techniques. The Auramine stained smears are examined with fluorescent microscope (excitation wavelength 350 nm and emission wavelength 450 nm). This method may be useful for screening samples, but identification should be confirmed with more specific assays (Casemore, 1991; Guyot, Sarfati, and Derouin, 2012; Hanscheid, Cristino, and Salgado, 2008).

Although useful, approaches employing these stains can suffer from low specificity and/or sensitivity, particularly for samples containing small numbers of oocysts (Clark, 1999). In

addition none of these approaches make possible the identification of *Cryptosporidium* species. To increase sensitivity of these techniques, methods of concentration such as the immunomagnetic separation (IMS) (Figure 4A and B), sucrose gradient or flotation can be used (Chalmers and Katzer, 2013).

If the presence of *Cryptosporidium* is still suspected even in the presence of negative results, it is recommended to obtain at least 2 more stool specimens from the patients, collected on separate days over a 10 day period.

5.2. Immunological methods

Immunological methods can offer some advantages over light microscopy for the detection of *Cryptosporidium* oocysts and for the diagnosis of cryptosporidiosis. Like the staining methods, these approaches do not allow the identification of *Cryptosporidium* species. Despite the good specificity, the limitation of all the immunoassays is their lower sensitivity for the diagnosis of infections due to *Cryptosporidium* species other than *C. parvum* or *C. hominis*. The consequences of this lower sensitivity should be considered in certain populations in which non-*C. parvum* / *C. hominis* species are more widely represented (Cama et al., 2008; Cama et al., 2007). In fact, some cases of cryptosporidiosis would have been missed if these assays had been the only methods used for diagnosis. However, these tests could be useful when experienced stool microscopists are not available (Agnamey et al., 2011). Some of these methods are described below:

Enzyme-linked Immunoassays (EIA): It has been used more frequently for the detection of *Cryptosporidium* coproantigens in human and animal stool samples and in both cases with an increased sensitivity of detection. This test is highly standardized and reproducible to minimize variability and it can be correlated with IFA-confirmed microscopy (Chalmers et al., 2011a).

Immunofluorescent Assay (IFA): Actually this tool involves the use of fluorescence microscopy and the direct fluorescent antibody (DFA) assay using a fluorescein isothiocyanate-conjugated anti-*Cryptosporidium* monoclonal antibody (FITC-C-mAb) which recognizes surface epitopes on oocysts (Figure 4B). It has been reported to achieve relatively high specificities (96–100%) and sensitivities (98.5–100%) for the detection of *Cryptosporidium* oocysts in faecal smears and environmental samples (Garcia and Shimizu, 1997; Garcia, Shimizu, and Bernard, 2000; Garcia et al., 2003). The IFA kit is clearly superior to light microscopy (which is relatively inexpensive) and has an obvious advantage in terms

of sensitivity particularly for smears with low concentrations. Another advantage of IFA is the rapid and cost-effective screening of large numbers of faecal samples (Garcia et al., 2003).

Immunochromatography (IC): These tests use monoclonal antibodies directed against specific membrane proteins of *Cryptosporidium.* The method is a one-step lateral flow immunochromatographic assay containing specific antibodies labeled by colored latex particles to generate a visual signal. The test strip is immersed in the diluted sample and results are read after 5–10 min. Positive samples for *Cryptosporidium* give blue bands which appear along with green control bands (Weitzel et al., 2006).

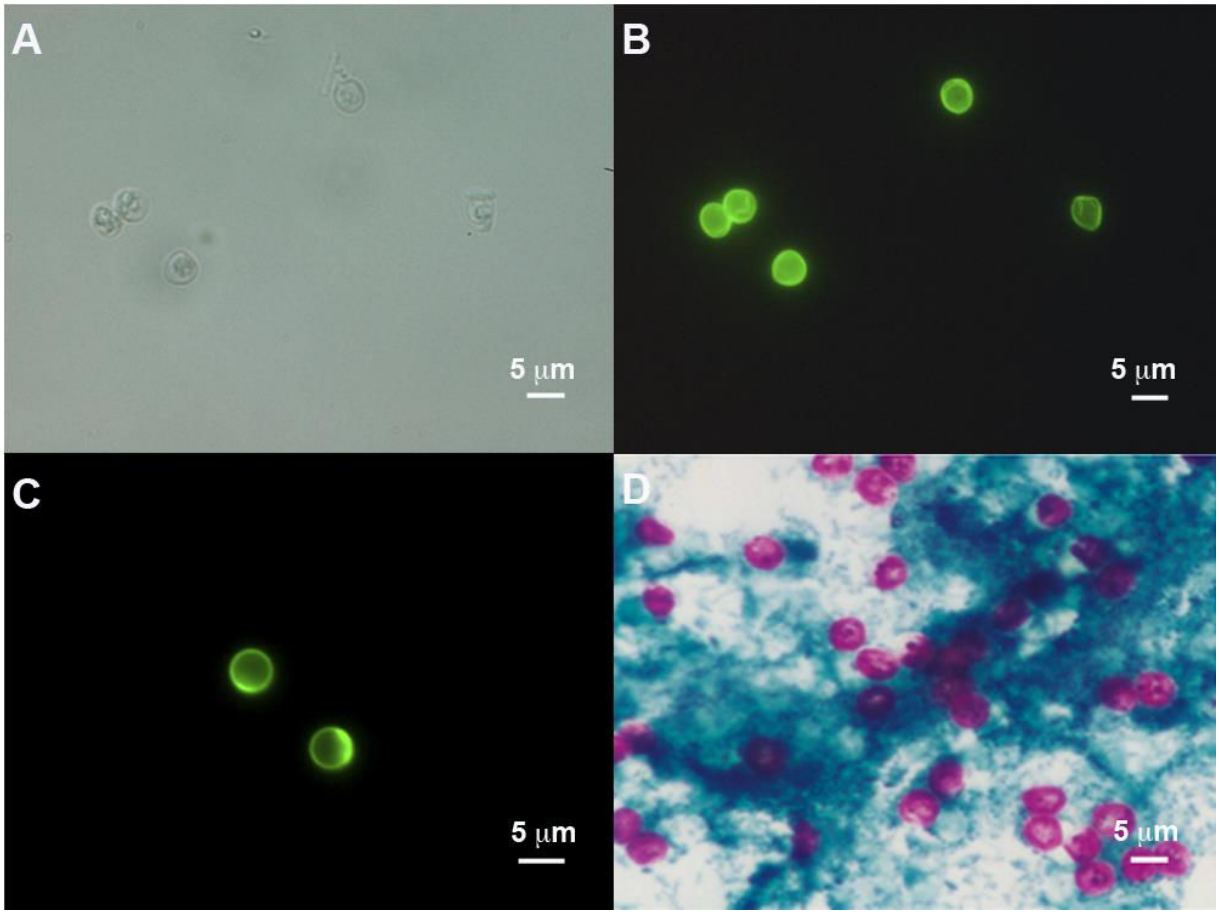


Figure 4. Microscopic observation of oocyts of *Cryptosporidium*. (A) Differential interference contrast image of oocysts recovered from stools using immunomagnetic separation (IMS). (B) Oocyts labeled by immunofluorescence (fluorescein) using a monoclonal antibody-based assay after recovering from stools using IMS. (C) Auramine staining of *Cryptosporidium* sp. oocysts. This photomicrograph was kindly provided by Dr. Emilie Frealle et Dr Yohann Le Govic from "Parasitologie-Mycologie, Centre Hospitalier Régional et Universitaire de Lille, Univ Lille Nord de France" (D) Modified Ziehl-Neelsen staining of *Cryptosporidium* sp. oocysts in a faecal smear.

5.3. Molecular tools

There are significant limitations in the specific diagnosis using microscopic, serological and biochemical techniques, hence the importance of molecular tools. In the recent years, several investigations have been conducted to develop and proceed to molecular characterization of isolates of *Cryptosporidium*. Even though these tests are commonly used in research laboratories, they are rarely used in medical laboratories because they are very expensive, very slow and require a professional technician. Polymerase Chain Reaction (PCR) is used in order to define parasite species/genotypes. The main limitations of using this technique are related to the extraction of DNA when the intensity of the infection is low in naturally infected hosts/environmental samples or when the extracted DNA contains PCR inhibitors (Paziewska et al., 2007). However, this technique does not provide data on the viability and infectivity of *Cryptosporidium* oocysts. To obtain additional information about these very important aspects, indirect methods, such as the Reverse-Transcriptase PCR (RT-PCR) must be used. Since RT-PCR is based on mRNA, which usually has a very short half-life, its use provides a more closely correlated indication of viability status compared to DNA-based methods (Skotarczak, 2010).

Different genetic markers have been used to amplify DNA by PCR from the purified oocysts (Table 3) for the detection and genotyping of *Cryptosporidium* but the molecular marker that is most often used for the detection of *Cryptosporidium* encodes the 18 small subunit (18-SSU) rRNA gene (Feng et al., 2007; Pedraza-Diaz et al., 2001; Xiao et al., 2001b).

Table 3. Selected genetic markers commonly used for the specific, genotypic or subgenotypic classification of species of *Cryptosporidium* for diagnostic, epidemiological and population genetic applications

Genetic marker or locus	Relative degree of variability	Tests	Main purposes
Small subunit (SSU) of nuclear ribosomal RNA	Low	Nested PCR, sequencing, PCR-RFLP, Real Time PCR, microarrays	Specific or genotypic identification (Systematics, diagnosis and epidemiology)
70 kDa heat shock protein (HSP70) gene	Low	Nested PCR, sequencing, PCR-RFLP, Real Time PCR, microarrays	Specific or genotypic identification (Systematics, diagnosis and epidemiology)
Actin gene	Low	Nested PCR, sequencing,	Specific or genotypic identification (Systematics, diagnosis and epidemiology)
***Cryptosporidium* oocyst wall protein (COWP) gene**	Low	Nested PCR, sequencing, PCR-RFLP, microarrays	Specific or genotypic identification (Taxonomy, diagnosis and epidemiology)
Microsatellite	High	Nested PCR, sequencing,	Genotypic and/or subgenotypic identification

		PCR-RFLP, Real Time PCR, microarrays	(Population genetic and epidemiological studies)
60 kDa glycoprotein (gp60) gene	High	Nested PCR, sequencing,	Genotypic and/or subgenotypic identification (Population genetic and epidemiological studies)

Data compiled from Jex et al., 2008.

The PCR–restriction fragment length polymorphism (RFLP) technique has been commonly used for the genetic classification of *Cryptosporidium* species and *C. parvum* genotype (Xiao et al., 2001b; Xiao et al., 1999). Sequence analysis of gene is widely used in *Cryptosporidium* subtyping because of its sequence heterogeneity and relevance to parasite biology. It is the most single polymorphic marker identified so far in the *Cryptosporidium* genome (Gatei et al., 2006a; Leoni et al., 2007; Wielinga et al., 2008).

The advent of real time PCR has enabled improved characterization of different species and genotypes of individual *Cryptosporidium* oocysts. Methods of quantitative real-time PCR using the TaqMan or the Applied Biosystems, technology have been used. In both cases, colorants fluorochromes are incorporated into the amplicon during the PCR; therefore, the fluorescence of the amplicon increases gradually as the product of amplification is generated. Both systems can detect PCR products during the first cycle of the reaction while the amplification is exponential, which allows a quantitative analysis of fluorescent products. As well, real time PCR procedures for the detection and genotyping of oocysts of *Cryptosporidium* provide a reliable, specific, and rapid detection method alternative to nested PCR, with a baseline sensitivity of between 1 and 10 oocysts (Fontaine and Guillot, 2002; Sunnotel et al., 2006). However, the relatively high cost of molecular methods at present has limited their application, especially in developing countries.

6. Treatment

Treatments modalities for cryptosporidiosis are limited. Usually, drug therapy is not needed for the treatment of immunocompetent persons but supportive therapy may be required to prevent dehydration and restore electrolyte balance, especially in children and pregnant women. In some cases rehydration salts are required (Chalmers and Katzer, 2013). Nitazoxanide has been FDA-approved in U.S.A. since 2004 for treatment of diarrhea caused by *Cryptosporidium* in immunocompetent persons ≥ 1 year of age (Marcos and Gotuzzo, 2013).

Table 4. Dosage used for Nitazoxanide

Adult dosage	500 mg BID x 3 days
Pediatric dosage	1-3 years: 100 mg BID x 3 days
	4-11 years: 200 mg BID x 3 days

BID : (*latin=bis in die*) Twice daily

In immunodeficient patients management, the most important is the improvement in the host's immune status (Marcos and Gotuzzo, 2013). Several molecules have been found to be partially effective for the treatment of cryptosporidiosis. In HIV-infected children with cryptosporidiosis, a randomized clinical trial using 28 days of nitazoxanide demonstrated no difference in eradication of infection or reduction of symptoms compared with placebo. Furthermore, a Cochrane review of several randomized clinical trials focusing on treatment or prevention of cryptosporidiosis in immunocompromised individuals concluded that no effective treatment is available for the management of cryptosporidiosis, but reduction in immunosuppression was strongly recommended. In HIV-infected individuals, a key step in controlling diarrhea associated with *Cryptosporidium* is the introduction of the highly active antiretroviral treatment (HAART) by reducing the immunosuppression (Marcos and Gotuzzo, 2013).

7. Control and prevention

The treatment options for cryptosporidiosis are limited. Then, prevention to reduce the risks factors of infection is the most important intervention. Some important measures intended to the prevention and control of the infection are highlighted in Table 5.

Table 5. Prevention to reduce risks factors of cryptosporidiosis

Risk factors of infection	Prevention
Consumption of untreated water	- Avoid drinking water or using ice resulting from a doubtful source - Drink potable or boiled water - Use of adapted filters
Consumption of fruits or vegetables contaminated with oocysts	- Washing of fruits and vegetables prior to consumption
Contact with infected animals (in particular, young calves (veal) (mainly a risk for *C. parvum*)	- Avoid the contact with animals susceptible to be contaminated - Wearing disposable gloves when cleaning animal feces - Hand washing with soap and water after the contact with animals - Quarantine of animals in facilities easy to clean and disinfect
Life in communities	- Hand washing - *Cryptosporidium* infected patients should be excluded from the work place, school or institutional settings
Diarrhea among other members of the household and changing diapers to young children	- Hand washing with soap and water - Proper disposal of excreta and washing of soiled materials such as clothing and bedding
Swimming	- To be warned by the risk of swimming in unchecked waters (lakes, rivers, leisure centers which can be contaminated) - Cases should avoid using swimming pools at least two weeks after diarrhea stops
Traveling abroad	- Hygiene recommendation to the travelers
Infection HIV/SIDA : CD4 < 200/mm3	- Follow all the hygiene recommendations mentioned above - Avoid close contact with any person or animal with cryptosporidiosis - Correction of the immunodeficiency

Data compiled from Ripert and Guyot 2013 and www.cdc.gov/parasites/prevention.html

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2. « Epidémiologie moléculaire de la cryptosporidiose au Moyen Orient ».

1. La cryptosporidiose

La cryptosporidiose est une maladie cosmopolite, avec une incidence et une prévalence variable selon les pays. La prévalence dépend d'une part de la région géographique, de la saison et du niveau socio-économique de la population étudiée et d'autre part de la virulence de la souche de *Cryptosporidium* présente et du statut immunitaire de la population ciblée. Actuellement, plus de 25 espèces ont été décrites pour le genre *Cryptosporidium*. Deux d'entre elles, *C. hominis* et *C. parvum*, sont responsables de plus de 95% des cas d'infections humaines (Ryan, Fayer, and Xiao, 2014).

Plusieurs études réalisées dans des pays développés et en développement ont permis d'identifier un certains nombres de facteurs de risque de cryptosporidiose: l'âge <5 ans, l'absence d'allaitement maternel, le contact avec les animaux, les mauvaises conditions de vie, le déficit immunitaire , la malnutrition et les co-infections (Putignani and Menichella, 2010). La distribution du parasite chez les humains et les animaux a également été attribuée à différentes sources d'infection et voies de transmission. La principale voie de contamination est la voie féco-orale. Elle peut être indirect en consommant un aliment ou une eau contaminée, ou direct par contact avec un hôte infecté. L'ingestion d'une quantité relativement faible d'oocystes suffit à induire une infection : la dose minimale infectante est de 9 oocystes chez des volontaires sains (Okhuysen et al., 1999). Un autre mode de transmission, via l'inhalation d'oocystes, a également été signalé mais plus rarement chez des individus immunodéprimés et des enfants (Sponseller, Griffiths, and Tzipori, 2014).

Le symptôme le plus généralement associé à l'infection par *Cryptosporidium* spp. est une diarrhée caractérisée par d'abondantes selles aqueuses (jusqu'à 10 fois/jour rarement accompagné de sang) qui peuvent être accompagnées d'une perte de poids rapide, de crampes abdominales, de nausées, de céphalées, de vomissements, de fièvre et de douleurs musculaires. Ces symptômes sont généralement auto-résolutifs dans les 2 semaines suivant l'infection, chez un individu adulte immunocompétent. Les enfants de moins de 2 ans, les personnes âgées et les patients immunodéprimés sont plus affectés et présentent les diarrhées les plus sévères (Leitch and He, 2012). Des données récentes de l'Etude Mondiale Entérique Multicentrique (Global Enterics Multi-Center Study-GEMS) sur le fardeau et l'étiologie de la diarrhée infantile dans les pays en développement ont montré que la cryptosporidiose est la

deuxième cause de diarrhée modérée à sévère et de mortalité chez le nourrisson (Kotloff et al., 2013; Striepen, 2013).

Les oocystes de *Cryptosporidium* sont largement présents dans les écosystèmes aquatiques et pourraient être détectés dans 87% des échantillons d'eau non traitée (LeChevallier, Norton, and Lee, 1991). La capacité de résistance aux différents mécanismes d'inactivation physiques ou chimiques a permis à ce protozoaire d'être responsable de plus de 165 épidémies d'origine hydrique (Chalmers, 2012) dont celle de Milwaukee en 1993, qui est la plus connue car elle a contaminé près de 403 000 personnes (Mac Kenzie et al., 1994). L'Organisation Mondiale de la Santé (OMS) a considéré ce parasite comme un « agent pathogène de référence » reflétant la qualité de l'eau. C'est à dire qu'il fait partie des protozoaires transmis par voie oro-fécale pris en compte dans la conception et la mise en œuvre des recommandations pour la qualité de l'eau potable (WHO Guidelines for Drinking Water Quality) (WHO, 2011). De même, les épidémies d'origine alimentaire dues à *Cryptosporidium* spp. ont considérablement augmenté durant la dernière décennie (Putignani and Menichella, 2010). Tous ces éléments conduisent à considérer la cryptosporidiose comme un problème de santé publique majeur d'où l'importance de contrôler sa présence dans l'environnement (WHO, 2006).

De plus, le statut d'agent nocif trouve une nouvelle justification depuis l'ajout en 2004 de la cryptosporidiose sur la liste de « The WHO Neglected Diseases Initiative », programme regroupant un ensemble de maladies parasitaires, bactériennes et virales qui constituent un frein au développement socio-économique dans les pays les plus pauvres et pour lesquelles des études de terrain sont indispensables à leur compréhension et à l'établissement de conduites permettant leur contrôle.

D'autre part, la fréquence des infections à *Cryptosporidium* spp. varie suivant les variations saisonnières dans la plupart des pays. Ces fluctuations se produisent à des moments différents selon la région géographique et sont basées probablement sur le risque de contamination de l'eau potable. Dans les pays développés, un pic annuel est fréquemment observé en été. Période pendant laquelle les personnes fréquentent plus souvent les piscines et les lacs (Fournet et al., 2013). Par contre, dans les pays tropicaux, la plus haute prévalence de la cryptosporidiose est généralement associée à la saison d'hiver où l'on constate une augmentation des précipitations (Iqbal, Khalid, and Hira, 2011). Certains pays comme le Royaume-Uni peuvent avoir deux pics (au printemps et en automne). Il a été rapporté que le pic de printemps est principalement dû à *C. parvum* tandis que le pic d'automne est

principalement dû à *C. hominis*, ce qui suggère que les modalités de transmission de l'infection varient également en fonction de la saison (Chalmers et al., 2009).

2. **Le besoin d'utiliser des outils moléculaires pour la détection du parasite**

La cryptosporidiose, peut être anthroponotique (contamination par un humain infecté) ou zoonotique (contamination par un animal infecté). Les espèces *C. hominis* et *C. viatorum* ainsi que la famille de sous-type *C. parvum* IIc sont transmis exclusivement par la voie anthroponotique (Ryan, Fayer, and Xiao, 2014). De rares exceptions ont été signalées chez le bétail et d'autres animaux (Smith et al., 2005). En revanche, d'autres espèces tels que *C. parvum* (à l'exception de la famille de sous-type *C. parvum* IIc), *C. meleagridis*, *C. canis*, *C. felis*, *C. cuniculus*, *C. andersoni* et *C. bovis* ont été isolées aussi bien chez l'homme que chez les animaux (Chalmers and Katzer, 2013).

Dans les pays développés et certains pays en développement, la majorité des enquêtes épidémiologiques sur la cryptosporidiose ont ciblé la détection et la caractérisation génétique des isolats du parasite par des outils moléculaires. L'utilisation de ces méthodes reste toujours la meilleure technique pour mesurer la prévalence de la cryptosporidiose et étudier la diversité génétique des isolats dans des échantillons humains, d'animaux et environnementaux, ce qui peut nous donner une idée des différentes voies de transmission et de circulation du parasite (Chalmers and Katzer, 2013; Jex et al., 2008b). Cependant, les voies de transmission de la cryptosporidiose ne sont pas entièrement claires, surtout dans les pays en développement. Cela est dû au fait que la majorité des études réalisées dans ces pays sont basées sur des techniques de diagnostiques qui ne permettent pas une différenciation entre les sources d'infections (Nazemalhosseini-Mojarad, Feng, and Xiao, 2012).

Différentes techniques moléculaires de détection sont disponibles à l'heure actuelle. La réaction en chaîne par polymérase (PCR) est généralement utilisée dans le but d'identifier les espèces / sous-types parasitaires. Le gène (ADNr 18S) codant l'ARNr 18S est le marqueur moléculaire le plus souvent utilisé pour l'identification de *Cryptosporidium* (Xiao et al., 1999). D'autres marqueurs existent comme la protéine de la paroi des oocystes (COWP) (Pedraza-Diaz et al., 2001), la protéine chaperonne (HSP-70) (Morgan et al., 2000), la glycoprotéine 60 kDa (gp60) (Alves et al., 2003), la bêta-tubuline (Tanriverdi et al., 2002) et le locus de microsatellite 1 (ML1) et 2 (ML2) (Chalmers et al., 2005; Hunter et al., 2007; Leoni et al., 2007). Par contre, l'étude de ces marqueurs ne fournit pas de données sur la viabilité et la capacité infectieuse des oocystes de *Cryptosporidium*. Pour obtenir des

informations supplémentaires sur ces aspects très importants, des méthodes indirectes, comme la transcriptase inverse - PCR (RT-PCR), doivent être utilisés (Skotarczak, 2010). D'autre part, la RFLP (Polymorphisme de longueur des fragments de restriction) permet le génotypage des isolats et la mise en évidence des espèces de *Cryptosporidium* chez l'homme, les animaux et les échantillons environnementaux. Le principe est basé sur l'utilisation des enzymes de restrictions SspI et VspI suite à la PCR ciblant le fragment du gène ADNr 18S (Xiao et al., 1999). Pour l'analyse des échantillons provenant des ruminants, une optimisation de la méthode a été faite par Feng et al. Le procédé a été modifié en utilisant les deux enzymes SspI et MboII dans l'analyse de la RFLP (Feng et al., 2007). Cependant le séquençage de l'ADN des produits de PCR amplifiés reste l'outil le plus couramment utilisé actuellement (Koinari et al., 2013; Ryan, Fayer, and Xiao, 2014).

De plus, le marqueur moléculaire gp60 permet le typage de souches de *Cryptosporidium* et l'identification des sous-types des espèces : *C. hominis*, *C. parvum*, *C. meleagridis*, *C. cuniculus*, *C. wrairi*, *C. tyzzeri* et *C. ubiquitum* et d'autres génotypes : horse genotype, ferret genotype, mink genotype et rabbit genotype. Cette protéine, dont le gène à un haut degré de polymorphisme de séquence, est située sur la surface de la région apicale des stades invasifs du parasite, et constitue une des cibles dominantes dans la réponse immunitaire humorale chez l'homme. Cette caractérisation génétique est nécessaire pour l'évaluation du potentiel anthroponotique / zoonotique de chaque sous-type et ainsi que pour mieux comprendre la dynamique de la transmission de cette maladie. Les sous-types séquencés seront nommés en comptant le nombre de répétitions des trinucléotides : TCA (A), TCG (G), et TCT (T) et d'autres répétitions : AA/GGACGGTGGTAAGG (R) pour *C. hominis* et ACATCA (R) pour *C. parvum* (Tableau 1) (Alyousefi et al., 2013). Néanmoins, la PCR ciblant le gène gp60 a une sensibilité inférieure à celle ciblant l'ADNr 18S ce qui nécessite des échantillons fortement chargées en oocystes pour une bonne identification des sous-types (Chalmers et al., 2005).

Tableau 1: Familles majeures de sous-types de *Cryptosporidium* chez les mammifères (Feng et al., 2011; Nichols, Chalmers, and Hadfield, 2014)

Familles de sous-types de *C. hominis*	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
Ia	TCA	AA/GGACGGTGGTAAGG	IaA18R3
Ib	TCA, TCG, TCT	---	IbA10G2
Id	TCA, TCG	---	IdA15G1
Ie	TCA, TCG, TCT	---	IeA11G3T3
If	TCA, TCG	---	IfA19G1
Ig	TCA	---	IgA24
Ih	TCA, TCG	---	IhA14G1
Ii (*C. hominis* monkey genotype)	TCA	---	IiA17
Ij	TCA	---	IjA14
Familles de sous-types de *C. parvum*	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
Iia	TCA, TCG	ACATCA	IIaA15G2R1
IIb	TCA	---	IIbA12
IIc	TCA, TCG	---	IIcA5G3
IId	TCA, TCG	---	IIdA20G1
IIe	TCA, TCG	---	IIeA7G1
IIf	TCA	---	IIfA6
IIg	TCA	---	IIgA9
IIh	TCA, TCG	---	IIhA7G4
Iii	TCA	---	IIiA10
IIk	TCA	---	IIkA14
IIl	TCA	---	IIlA18
IIm	TCA, TCG	---	IImA7G1
IIn	TCA	---	IInA8
IIo	TCA, TCG	---	IIoA16G1
Familles de sous-types de *C. meleagridis*	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
IIIa	TCA, TCG	---	IIIaA24G3
IIIb	TCA, TCG	---	IIIbA26G1R1
IIIc	TCA	---	IIIcA6
IIId	TCA	---	IIIdA6
IIIe	TCA, TCG	---	IIIeA20G1
Familles de sous-type de *C. fayeri*	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
IVa	TCA, TCG, TCT	---	IVaA11G3T1
IVb	TCA, TCG, TCT	---	IVbA9G1T1

IVc	TCA, TCG, TCT	---	IVcA8G1T1
IVd	TCA, TCG, TCT	---	IVdA7G1T1
IVe	TCA, TCG, TCT	---	IVeA7G1T1
IVf	TCA, TCG, TCT	---	IVfA12G1T1
Familles de sous-types de *C. cuniculus*	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
Va	TCA	---	VaA18
Vb	TCA	---	VbA29
Familles de sous-types de Horse genotype	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
VIa	TCA, TCG	---	VIaA11G3
VIb	TCA	---	VIbA13
Familles de sous-type de *C. wrairi*	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
VIIa	TCA, TCT	---	VIIaA17T1
Familles de sous-types de Ferret genotype	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
VIIIa	TCA, TCG	---	VIIIaA5G2
Familles de sous-types de *C. tyzzeri*	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
IXa	TCA	A/GTTCTGGTACTGAAGATA	IXaA6R3
IXb	TCA	---	IXbA6R2
Familles de sous-types de Mink genotype	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
Xa	TCA, TCG	---	XaA5G1
Familles de sous-types de Opossum genotype	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
XIa	TCA, TCG, TCT	---	XIaA4G1T1
Familles de sous-types de *C. ubiquitum*	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
XIIc	---	---	---
Familles de sous-types de Hedgehog genotype	**Trinucléotides répétés dominants**	**Autres répétitions**	**Exemples**
XIIIa	TCA	ACATCA	XIIIaA22R9

3. *Cryptosporidium* chez l'homme dans les pays développés et en développement

Plus de 150 études publiées dans des bases de données MEDLINE / PubMed ont été examinées. Malgré l'importance mondiale que *Cryptosporidium* a prise durant la dernière décennie, nous avons noté un manque d'informations sur la transmission du parasite dans plusieurs pays de l'Asie, l'Afrique et L'Amérique du Sud. Cependant, le nombre d'études épidémiologiques sur le terrain est en augmentation significative ces dernières années. Par contre, alors que la stratégie de recherche dans les pays développés a été d'étudier la diversité génétique des isolats de *Cryptosporidium*, la majorité des pays en développement se sont concentrés sur des études épidémiologiques recherchant simplement la prévalence de la cryptosporidiose (Tableau 2). Les données disponibles montrent que *Cryptosporidium* spp. est en moyenne, 5 à 20 fois plus répandue chez la population humaine dans les pays en développement que dans les pays développés. La prévalence de l'infection varie de 0 à 2% dans les pays industrialisés et peut atteindre 50% dans les pays en développement [14]. De plus, la plupart des études publiées dans les pays en développement utilisent des techniques microscopiques ou immunologiques dont la faible sensibilité peut largement sous-estimer la prévalence réelle de la cryptosporidiose (Tableau 2). En effet, une étude réalisée en Jordanie a montré que la prévalence de la cryptosporidiose était dix fois plus élevée lorsque le parasite était détecté en utilisant la PCR en temps réel ciblant l'ADNr 18S comparativement à ce qui a été obtenu par microscopie (Hijjawi et al., 2010). Une autre étude réalisée en Egypte a montré que la prévalence de la cryptosporidiose était de 6,7% par la technique immunologique Copro-Antigène RIDA® rapide et qu'elle augmentais à 50% lorsque cette méthode était remplacée par la PCR en temps réel (Helmy et al., 2013). D'autre part une variabilité de sensibilité et de spécificité peut être détectée en utilisant différentes méthodes phénotypiques. Une étude en Turquie a montré une augmentation de la prévalence de 5,2% à 24% lorsque l'on passait de la microscopie à l'ELISA (Elgun and Koltas, 2011).

Les deux espèces *C. hominis* et *C. parvum* ont été décrites comme étant prédominantes dans la quasi-totalité des études publiés dans le monde. *C. hominis* prédomine dans la majorité des pays tropicaux et d'Asie de l'Est. Alors que *C. parvum* prédomine dans la plupart des pays industrialisés et dans les pays du Moyen Orient (Tableau 2).

L'étude de la diversité génétique des souches de *Cryptosporidium* isolées montre que les familles de sous-types *C. hominis* Ib, Id et Ie, *C. parvum* IIa et IId et *C. meleagridis* IIIb ont la prévalence la plus importante dans la population humaine. À ce jour et à notre

connaissance, les deux sous-types *C. hominis* IbA10G2 et *C. parvum* IIaA15G2R1 sont les plus prédominants dans le monde (Tableau 2). Cependant, la virulence et l'impact clinique de divers sous-types de *C. hominis* chez l'homme n'est pas encore clair. Les enquêtes ont cependant montré la présence d'une probable association entre *C. hominis* et des symptômes digestifs plus sévères dans les cas d'infection par certains sous-types tels que le sous-type IbA10G2 (Cama et al., 2008; Li et al., 2013). De plus, ce sous-type a été rapporté comme étant responsable de la majorité des épidémies liées à *Cryptosporidium* en Europe et aux États-Unis dont l'épidémie de Milwaukee en 1993 (Xiao, 2010). Quant au sous-type *C. parvum* IIaA15G2R1, il est largement répandu chez les animaux, notamment chez les bovins. Des études récentes ont même permis de mettre en évidence son potentiel tumorigène puisqu'il induit des adénocarcinomes digestifs chez un modèle murin immunodéprimé (Benamrouz et al., 2012; Certad et al., 2012).

4. *Cryptosporidium* chez l'animal dans les pays développés et en développement

Cryptosporidium est décrit chez de nombreux animaux, aussi bien, domestiques, sauvages qu'en captivité. Les animaux les plus jeunes semblent être les plus susceptibles et les plus touchés par la maladie (Ramirez, Ward, and Sreevatsan, 2004).

L'intérêt pour l'infection due à *Cryptosporidium* chez l'animal s'est intensifié à mesure que les cas d'infections humaines et animales ont augmentées, non seulement parce que les animaux ont été considérés comme des réservoirs, mais également parce que l'infection par ce protiste peut avoir des répercussions économiques chez les animaux de rente (Ramirez, Ward, and Sreevatsan, 2004). La cryptosporidiose du bétail est aujourd'hui particulièrement surveillée en raison des préjudices économiques qu'elle peut entraîner (de Graaf et al., 1999).

Les bovins sont souvent infectés par quatre espèces de *Cryptosporidium* : *C. parvum*, *C. andersoni*, *C. bovis* et *C. ryanae*. Des travaux ont montré que la prédominance d'une espèce est liée à l'âge de la population hôte. L'espèce *C. parvum* est retrouvée principalement chez les veaux pré-sevrés (Age < 8 semaines), *C. bovis* et *C. ryanae* chez les veaux sevrés, et *C. andersoni* chez les bovins adultes (Follet et al., 2011; Santin et al., 2004). C'est pourquoi, on estime que les bovins peuvent jouer un rôle clé dans la transmission de *C. parvum* à potentiel zoonotique. Des études génétiques plus approfondies, ciblant le marqueur gp60 ont montré que la majorité des isolats de *C. parvum* décrits appartiennent aux familles de sous-type IIa et IId. La famille de sous-type *C. parvum* IIa a été décrite comme étant prédominante dans toutes les études réalisées chez le bétail dans le monde, à l'exception d'une étude en Egypte

qui a rapporté une prédominance de la famille de sous-type *C. parvum* IId (Tableau 3). Ces familles de sous-type, isolées chez les humains et les ruminants, sont responsables de la cryptosporidiose à transmission zoonotique. La majorité des études dans le monde ont signalés une prédominance du sous-type *C. parvum* IIaA15G2R1 chez les bovins. Cependant, un autre sous-type, *C. parvum* IIaA18G3R1, a été rapporté comme prédominant en Irlande du Nord et en Australie (Tableaux 2 et 3).

La famille de sous-type *C. parvum* IId a été précédemment rapportée chez les bovins en Iran (Nazemalhosseini-Mojarad et al., 2011), en Tunisie (Rahmouni et al., 2014), au Portugal (Alves et al., 2006b), en Roumanie (Imre et al., 2013), en Hongrie (Plutzer and Karanis, 2007) et en Espagne (Quilez et al., 2008). Ce sous-type n'a cependant, jamais été décrit chez l'homme ou chez les bovins en Australie, aux États-Unis et au Canada (Tableaux 2 et 3).

Comme chez l'homme, certaines infections dues à *Cryptosporidium* chez les bovins peuvent être asymptomatiques, en particulier dans les zones endémiques des pays en développement. Par conséquent, la protection de cette population vulnérable est importante pour la prévention de la transmission de la maladie.

Certains animaux domestiques tels que les oiseaux, les cochons, les chiens et les chats présentent un risque plus faible pour la transmission de la cryptosporidiose. Ces derniers sont majoritairement infectés par des espèces autres que *C. hominis* et *C. parvum* et n'ayant pas un fort degré de virulence pour l'homme immunocompétent (Xiao, 2010).

Cryptosporidium a été aussi détecté chez des animaux sauvages tels que les souris, les cerfs, les sangliers, les lézards, les renards, les serpents, etc. (Ryan, 2014). Chez tous ces animaux, les infections ont été décrites comme asymptomatiques. Cependant, certains animaux sauvages, particulièrement les rongeurs, partagent aussi leurs habitats avec l'homme et les animaux de rente, fournissant une source supplémentaire de contamination de l'environnement et d'infection pour le bétail (Ramirez, Ward, and Sreevatsan, 2004).

Les poissons peuvent également être infectés par *Cryptosporidium*, cependant l'épidémiologie moléculaire de la cryptosporidiose chez ces animaux n'est pas bien connue. Peu d'informations sont disponibles sur la prévalence et de la diversité génétique des espèces de *Cryptosporidium* dans les milieux aquatiques et le rôle de ces animaux dans la transmission de la maladie à l'homme (Ryan, Fayer, and Xiao, 2014).

5. La cryptosporidiose au Moyen Orient

Les pays du Moyen Orient, à l'instar de la plupart des pays en développement, sont très touchés par la cryptosporidiose. La situation de la cryptosporidiose chez l'homme dans ces pays commence à s'éclaircir suite au nombre important d'études de terrain réalisées au cours de la dernière décennie (Alyousefi et al., 2013; Helmy et al., 2013; Hijjawi et al., 2010; Iqbal, Khalid, and Hira, 2011; Nazemalhosseini-Mojarad et al., 2011; Sulaiman et al., 2005; Zavvar et al., 2008). Ces investigations ont souvent aidé les chercheurs à clarifier les modes de transmission ainsi que les facteurs de risques liés à *Cryptosporidium* spp. Néanmoins, ils manquent encore des données dans plusieurs pays tels que la Syrie, le Qatar, Oman, le Bahreïn ou le Liban. De plus, les techniques traditionnellement utilisées pour le dépistage de *Cryptosporidium* ne permettent pas d'étudier la diversité génétique des isolats. Les données de la cryptosporidiose chez les animaux et dans l'environnement quant à elles sont très rares, voir absente, dans cette région.

Plus de 80 études réalisées dans 10 états du Moyen Orient ont été publiées à ce jour. La cryptosporidiose y est décrite comme un problème majeur de santé publique dans la région. Le pic saisonnier de la cryptosporidiose est variable suivant les pays. Il est décrit pendant la saison d'été en Egypte (Abd El Kader et al., 2012), la saison d'automne en Iran (Mirzaei, 2007) et la saison d'hiver au Koweït (Iqbal, Khalid, and Hira, 2011). De même, la prévalence de la cryptosporidiose chez l'homme varie selon les pays. La prévalence la plus faible a été rapportée chez les enfants en Iran (2.4%) (Taghipour et al., 2011) et chez la population générale au Koweït (3.4%) (Iqbal, Khalid, and Hira, 2011). Par contre, l'utilisation des techniques microscopiques sous-estime la prévalence réelle du parasite dans ces pays ce qui rend la comparaison des données produites dans cette région difficile.

A notre connaissance, peu d'études ont été réalisées dans cette région en utilisant des outils moléculaires pour la recherche de *Cryptosporidium* chez l'homme (Alyousefi et al., 2013; Helmy et al., 2013; Hijjawi et al., 2010; Zavvar et al., 2008). Des travaux réalisés chez des patients hospitalisés ont montré des prévalences variables selon les pays: 10% chez les enfants et les adultes au Yémen (Alyousefi et al., 2013), 19% chez les enfants en Jordanie (Hijjawi et al., 2010), 49% chez les enfants en Egypte (Helmy et al., 2013) et 60% chez les patients sidéens en Iran (Zavvar et al., 2008).

C. parvum est l'espèce prédominante dans la plupart des pays de cette région géographique à l'exception de l'Egypte où l'espèce dominante est *C. hominis* (Alyousefi et al., 2013; Helmy

et al., 2013; Hijjawi et al., 2010; Iqbal, Khalid, and Hira, 2011; Usluca and Aksoy, 2011).Malgré le nombre élevé de sous-types et de familles d'allèles de *C. parvum* décrit dans ces pays, la plupart des isolats identifiés appartiennent à deux familles de sous-type, IIa et IId. Les sous-types *C. parvum* IIaA15G2R1 et IIdA20G1 sont les sous-types les plus rapportés chez l'homme et les bovins dans les pays du Moyen Orient. Il est à noter que le sous-type *C. parvum* IIdA20G1, fréquemment rencontré dans les pays du Moyen Orient, a été très peu rapporté dans d'autres pays du monde (Hijjawi et al., 2010; Imre et al., 2013; Nazemalhosseini-Mojarad et al., 2011; Sulaiman et al., 2005).

De même, plusieurs familles de sous-types de *C. hominis* ont été identifiées tels que Ia, Ib, Id, Ie et If. La famille anthroponotique de *C. parvum*, IIc, a également été identifiée dans cette région, mais en faible proportion en Jordanie et au Koweït (Hijjawi et al., 2010; Iqbal, Khalid, and Hira, 2011; Sulaiman et al., 2005).

En conclusion, la prévalence de la cryptosporidiose humaine reste mal évaluée et probablement sous-estimée au Moyen Orient. À ce jour, les données publiées ne suffisent pas à clarifier et comprendre la situation de la cryptosporidiose dans cette région. D'autres études moléculaires complémentaires, y compris sur un grand nombre d'échantillons humains, animales et environnementaux, sont indispensables pour une meilleure compréhension du dynamisme de la transmission de ce parasite.

La mise en place de bonnes mesures de prévention contre cette maladie nécessite une bonne compréhension de son épidémiologie et de son mode de transmission. Cette prévention doit être à la fois individuelle et collective basée sur l'hygiène et la protection des ressources d'eau contre une contamination environnementale par des oocystes de *Cryptosporidium* spp.

Tableau 2: Données récentes sur l'épidémiologie moléculaire de la cryptosporidiose dans différents pays du monde distribuées par continente

1. Afrique

Pays	Nombre d'échantillons analysés	Type d'étude : Détermination de prévalence (%) ou du génotypage	Population étudiée (Technique utilisée pour la détection)	Espèce de *Cryptosporidium* identifiée (%)	Sous-type prédominant	Autres sous-types	Référence
Égypte	200	60.2%	Enfants immunodéprimés (MZN)	ND	ND	ND	(Abdel-Hafeez et al., 2012)
	250	42.2%	Enfants (MZN)				
Égypte	165	49.1%	Enfants (PCR)	*C. hominis* (61%)	Problèmes pour le typage de *C. hominis*	IIdA20G1 IIaA15G1R1 IIaA15G2R1	(Helmy et al., 2013)
Égypte	391	5.9%	Enfants (MZN)	*C. hominis* (67%)	ND	ND	(Abd El Kader et al., 2012)
Ethiopie	378	8.4%	Sidéens (MZN)	ND	ND	ND	(Adamu, Wegayehu, and Petros, 2013)
Ethiopie	520	26.9%	Sidéens (PCR)	*C. parvum* (66%)	IIaA15G2R1	IIaA16G3R1 IIbA12 IIcA5G3 IIdA22G1 IdA20 IbA10G2 IeA11G3T3 IaA22R3 IeA11G3T3 IIcA5G3	(Adamu et al., 2014)
Madagascar	215	5.6%	Enfants (MZN)	*C. hominis* (91%)	IdA15G1		(Areeshi et al., 2008)
Sénégal	375	4.5%	Enfants (MZN)	ND	ND	ND	(Faye et al., 2013)
		6.1%	Enfants (ELISA)				
Afrique du Sud	244	18%	Générale (qPCR)	*C. hominis* (82%)	ND	ND	(Samie et al., 2006)
Tunisie	633	1.7%	Enfants (MZN)	*C. parvum* (45%)	ND	ND	(Essid et al., 2008)
	75	10.7%	Sidéens (MZN)				
Tunisie	403	1.7%	Enfants (MZN)	*C. parvum* (57%)	IIaA15G2R1	IIdA16G1	(Rahmouni et al., 2014)
Uganda	108	32.4%	Non spécifiée (PCR)	ND	ND	ND	(Salyer et al., 2012)

2. Asie de l'Ouest

Pays	Nombre d'échantillons analysés	Type d'étude : Détermination de prévalence (%) ou du génotypage	Population étudiée (Technique utilisée pour la détection)	Espèce de *Cryptosporidium* identifiée (%)	Sous-type prédominant	Autres sous-types	Référence
Iraq	205	9.7%	Diarrhéiques (MZN)	ND	ND	ND	(Mahdi and Ali, 2004)
Iran	794	2.4%	Enfants (MZN)	ND	ND	ND	(Taghipour et al., 2011)
Iran	150	16%	Enfants (Auramine O)	ND	ND	ND	(Ranjbar-Bahadori et al., 2011)
Iran	15	Génotypage	Non spécifiée (MZN)	*C. parvum* (73%)	ND	ND	(Meamar et al., 2007)
Iran	25	Génotypage	Enfants (MZN)	*C. parvum* (88%)	IIdA20G1	IIaA15G2R1 IIdA18G1	(Nazemalhosseini-Mojarad et al., 2011)
Iran	371	4.1%	Immunodéprimés (MZN)	*C. parvum* (69%)	ND	ND	(Rafiei et al., 2014)
Jordan	1585	1.8%	Enfants (MZN)	*C. parvum* (50%)	IIdA20G1	IbA6G3 IbA9G3 IdA24 IIaA15G1R1	(Hijjawi et al., 2010)
Jordan	104	19%	Enfants (qPCR)	ND	ND	ND	(Hijjawi et al., 2010)
Jordan	300	37.3%	Enfants (IF)	ND	ND	ND	(Mahgoub, Almahbashi, and Abdulatif, 2004)
KSA	253	11.5%	Enfants (MZN)	ND	ND	ND	(Al-Braiken et al., 2003)
KSA	35	Génotypage	Enfants (MZN)	*C. parvum* (43%)	ND	ND	(Al-Brikan et al., 2008)
KSA	100	11%	Enfants (MZN)	*C. parvum* (100%)	ND	ND	(Shalaby et al., 2014)
Kuwait	62	Génotypage	Enfants (MZN)	*C. parvum* (95%)	IIdA20G1	IIaA15G2R1 IIaA15G1R1 IbA10G2 IbA9G3	(Sulaiman et al., 2005)
Kuwait	2548	3.4%	Enfants (MZN)	*C. parvum* (73%)	IIa	IId, IIc, Id, Ia	(Iqbal, Khalid, and Hira, 2011)
Palestine	760	11.6%	Enfants (MZN)	ND	ND	ND	(Abu-Alrub et al., 2008)
Palestine	30	Génotypage	Enfants (MZN)	*C. parvum* (100%)	ND	ND	(Hussein, 2011)
Yemen	712	34.7%	Enfants (MZN)	ND	ND	ND	(Al-Shamiri, Al-Zubairy, and Al-Mamari, 2010)
Yemen	335	9.9%	Non spécifiée (PCR)	*C. parvum* (97%)	IIaA15G2R1	IeA11G3T3	(Alyousefi et al., 2013)

3. Asie de l'Est et l'Australie

Pays	Nombre d'échantillons analysés	Type d'étude : Détermination de prévalence (%) ou du génotypage	Population étudiée (Technique utilisée pour la détection)	Espèce de *Cryptosporidium* identifiée (%)	Sous-type prédominant	Autres sous-types	Référence
Australie	62	Génotypage	Non spécifiée (MZN)	*C. hominis* (61%)	IbA10G2R2	IbA9G3R2 IaA17R1 IfA12G1R2 IIaA18G3R1 IIaA20G3R1 IIaA22G4R1 IIcA5G3R2	(Jex et al., 2008a)
Australie	248	Génotypage	Non spécifiée	*C. hominis* (79%)	IdA15G1	IgA17 IdA12G1 IeA11G3T3 IIaA18G3R1 IIaA17G2R1	(Ng, MacKenzie, and Ryan, 2010)
Bangladesh	53	Génotypage	Enfants (MZN)	*C. hominis* (91%)	IeA11G3T3	IfA13G1 IbA10G2 IaA12G1R1 IaA21G1R1 IdA15G1 IdA24	(Hira et al., 2011)
Chine	10	Génotypage	Non spécifiée	*C. hominis* (91%)	IbA20G2	IImA7G1 IaA9R3 IbA16G2 IbA19G2	(Wang et al., 2011)
Chine	683	1.5%	Sidéens (PCR)	*C. meleagridis* (50%)	IIIbA26G1R1	IdA21 IbA19G2 IIdA19G1	(Wang et al., 2013)
Inde	970	4.5%	Sidéens (MZN)	*C. hominis* (67%)	IeA11G3T3	IaA18R3 IaA19R3 IaA21R3 IaA26R3 IaA27R3 IaA29G1T3R3 IdA14G1 IdA15G11	(Sharma et al., 2013)
	200	0.5%	Immunocompétents (MZN)	*C. hominis* (60%)		IdA16G1 IeA11G3T2 IfA13G1 IIcA5G3 IIdA14G1 IIdA15G1	
	130	3.8%	Enfants (MZN)	*C. hominis* (100%)		IIeA7G1	
Indonésie	318	11.3%	Sidéens (MZN)	*C. hominis* (82%)	ND	ND	(Kurniawan et al., 2013)
Japon	5	Génotypage	Non spécifiée	*C. hominis* (60%)	Ia, Ib, Id	IIa, IIc	(Abe et al., 2006)
Malaisie	122	22.1%	Sidéens (MZN)	*C. parvum* (64%)	IIdA15G2R1	IbA10G2R2 IaA14R1	(Lim et al., 2011)
Malaisie	346	5.2%	Sidéens (PCR)	*C. parvum* (72%)	IIaA15G2R1	IIaA13G1R1 IIaA13G2R1 IIaA14G2R1 IIdA15G2R1 IbA10G2R2 IaA14R1	(Iqbal et al., 2012)
Malaisie	77	1.3%	Non spécifiée (MZN)	ND	ND	ND	(Sinniah et al., 2012)
Mongolie	138	5.1%	Enfants (PCR)	*C. parvum* (100%)	ND	ND	(Hong et al., 2014)
Nouvelle Zélande	421	Génotypage	Non spécifiée	*C. parvum* (53%)	ND	ND	(Learmonth et al., 2004)
Pakistan	50	40%	Immunodéprimés (Kinyoun)	ND	ND	ND	(Baqai, Anwar, and Kazmi, 2005)
Sri Lanka	138	5.7%	Enfants (MZN)	ND	ND	ND	(Sirisena et al., 2014)
Sri Lanka	125	3.2%	Chargé de l'élevage (IF)	ND	ND	ND	(Ehsan et al., 2015)
Thaïlande	46	28.7% 4.4%	Sidéens (MZN) Sidéens (PCR)	*C. parvum* (100%)	ND	ND	(Nuchjangreed et al., 2008)

4. Europe and Amérique du Nord

Pays	Nombre d'échantillons analysés	Type d'étude : Détermination de prévalence (%) ou du génotypage	Population étudiée (Technique utilisée pour la détection)	Espèce de *Cryptosporidium* identifiée (%)	Sous-type prédominant	Autres sous-types	Référence
Belgique	ND	Génotypage	Troubles digestives	*C. hominis* (54%)	IbA10G2	IIaA15G2R1	(Geurden et al., 2009)
Canada	10	Génotypage	Non spécifiée	*C. parvum* (60%)	IIaA17G2R1	IIaA15G2R1 IIaA16G3R1 IeA11G3T3 IaA19R3 IaA23R4 IdA19	(Trotz-Williams et al., 2006)
France	24915	1.6%	Non spécifiée (MZN/PCR)	*C. parvum* (54%)	ND	ND	(ANOFEL, 2010)
France	46	Génotypage	Non spécifiée	*C. parvum* (57%)	ND	ND	(Ngouanesavanh et al., 2006)
Italie	14	Génotypage	Non spécifiée (IFA)	ND	ND	IIa15G2R1 IIa18G3R1	(Drumo et al., 2012)
Irlande	199	Génotypage	Non spécifiée (Conventionnelle)	*C. parvum* (80%)	IIaA18G3R1	IbA10G2	(Zintl et al., 2009)
Pays-Bas	95	Génotypage	Non spécifiée	*C. hominis* (82%)	IbA10G2	-	(Fournet et al., 2013)
Portugal	40	Génotypage	Sidéens (MZN)	ND	IbA10G2	IIaA15G2R1 IIcA5G3b IIcA5G3a IIbA14 IIdA17G1 IIdA19G1 IIdA21G1 IIdA22G1 IaA19R3 IdA15 IeA11G3T3 IfA14G1	(Alves et al., 2006a)
Ecosse	1972	Génotypage	Non spécifiée (MZN)	*C. parvum* (48%)	ND	ND	(Chalmers and Pollock, 2012)
Ecosse	1139	Génotypage of *C. hominis*	Non spécifiée (MZN)	*C. parvum* (57%)	IIaA15G1R1	IaA14R3 IaA14R2 IaA25R3 IaA9G3 IbA10G2 IdA17 IdA24 IeA11G3T3	(Deshpande et al., 2014)
Slovénie	ND	Génotypage	ND	ND	IIaA15G2R1	IIaA15G2R1 IIdA17G1R1 IIdA19G1R1 IIcA5G3 IIlA16R2 IaA17R3 IbA10G2	(Soba and Logar, 2008)
Espagne	405	1%	Enfants (CpAg-ELISA)	*C. hominis* (100%) (1 isolate)	ND	ND	(Cardona et al., 2011)
Turquie	162	11.1%	Diarrhéiques (MZN)	*C. parvum* (95%)	ND	ND	(Usluca and Aksoy, 2011)
Turquie	154	24%	Diarrhéiques (CpAg-ELISA)	ND	ND	ND	(Elgun and Koltas, 2011)
Angleterre	2115	Génotypage	Non spécifiée	*C. hominis* (75%)	IbA10G2	-	(Fournet et al., 2013)
Angleterre	4509	Génotypage	Non spécifiée	*C. hominis* (57%)	ND	ND	(Chalmers et al., 2011)
Angleterre	55	Génotypage	Non spécifiée (conventionnelle)	*C. parvum* (79%)	ND	ND	(Leone et al., 2009)
USA	49	Génotypage	Non spécifiée (IFA)	*C. parvum* (90%)	IIaA15G2R2	IIaA15G2R1 IIaA16G1R1 IIaA16G2R1 IIaA17G4R2 IbA10G2	(Feltus et al., 2006)

5. Amérique Centrale et du Sud

Pays	Nombre d'échantillons analysés	Type d'étude : Détermination de prévalence (%) ou du génotypage	Population étudiée (Technique utilisée pour la détection)	Espèce de *Cryptosporidium* identifiée (%)	Sous-type prédominant	Autres sous-types	Référence
Brésil	59	10.1%	Sidéens (MZN)	*C. parvum* (100%)	ND	ND	(Assis et al., 2013)
Chili	245	7.3%	Sidéens (MZN)	*C. parvum* (50%)	ND	ND	(Neira et al., 2012)
	178	2.2%	Immunocompetent (MZN)	*C. hominis* (75%)	ND	ND	
Colombie	103	21.4	Sidéens (MZN)	*C. hominis* (50%)	ND	ND	(Navarro-i-Martinez et al., 2006)
Haïti	1529	10.3%	Troubles digestives (MZN)	*C. hominis* (69%)	ND	ND	(Raccurt et al., 2006)
	49	Génotypage	Patients hospitalisés	*C. hominis* (66%)	ND	ND	(Ngouanesavanh et al., 2006)
Jamaïque	35	Génotypage	Sidéens (Non spécifiée)	*C. hominis* (71%)	IbA10G2	IeA12G3T3 IIcA5G3	(Gatei et al., 2008)
Nicaragua	272	35.7%	Enfants (PCR)	*C. parvum* (100%)	ND	ND	(Munoz-Antoli et al., 2011)
Pérou	368	29.6%	Enfants (MZN)	*C. hominis* (70%)	IbA10G2	IeA11G3T3 IaA12R4 IaA14R6 IdA10 IIcA5G3	(Cama et al., 2008)
Venezuela	37	27%	Sidéens (MZN)	*C. hominis* (80%)	ND	ND	(Certad et al., 2006)

ND : Non déterminé

MZN : Coloration de Ziehl-Neelsen modifiée

Sidéens : Patients atteints du Syndrome d'Immunodéficience Acquise (SIDA)

CpAg-ELISA : Coproantigens-ELISA

IF : Immunofluorescence

Tableau 3: Données récentes sur la diversité génétique des sous-types de *C. parvum* isolés chez les bovins dans différents pays du monde.

Pays	Nombre d'isolats	Sous-type prédominant (%)	Autres sous-types	Reference (Date)
Pays développés				
Australie	13	IIaA18G3R1 (38%)	IIaA16G3R1 IIaA19G4R1 IIaA17G2R1 IIaA20G3R1 IIaA21G3R1	(Ng et al., 2008)
Canada	36	IIaA15G2R1 (28%)	IIaA17G2R1 IIaA16G3R1 IIaA16G2R1 IIaA16G1R1 IIaA18G3R1 IIaA13G2R1	(Trotz-Williams et al., 2006)
Angleterre	52	IIaA15G2R1 (69%)	IIaA17G1R1 IIaA16G3R1 IIaA18G1R1 IIaA19G1R1 IIaA14G2R1	(Brook et al., 2009)
France	51	IIaA15G2R1 (75%)	IIaA17G1R1 IIaA16G3R1 IIaA16G2R1 IIaA16G1R1 IIaA13G1R1	(Follet et al., 2011)
France	52	IIaA15G2R1 (88%)	IIaA16G3R1 IIaA19G2R1	(Rieux et al., 2013)
Irlande du Nord	215	IIaA18G3R1 (56%)	IIaA15G2R1 IIaA17G2R1 IIaA19G4R1 IIaA20G3R1 IIaA19G3R1 IIaA17G3R1 et autres	(Thompson et al., 2007)
USA	175	IIaA15G2R1 (77%)	IIaA11G2R1 IIaA15G2R2 IIaA17G2R1 IIaA18G2R1 IIaA19G2R1	(Xiao et al., 2007)
Pays en développement				
Chine	13	IIaA15G2R1 (62%)	IIaA16G2R1 IIaA14G1R1 IIaA14G2R1 IIaA16G3R1	(Mi et al., 2013)
Égypte	106	IIdA20G1 (81%)	IIaA15G2R1	(Helmy et al., 2013)
Iran	25	IIaA15G2R1 (88%)	IIaA16G3R1 IId A15G1	(Nazemalhosseini-Mojarad et al., 2011)
Sri Lanka	2	IIdA16G1 (100%)	-	(Ehsan et al., 2015)
Tunisie	16	IIaA15G2R1 (86%)	IIdA16G1	(Rahmouni et al., 2014)

3. « *Cryptosporidium* et cancer ».

Le cancer représente l'une des principales causes de morbidité et de mortalité dans le monde. On comptait en 2012, environ 32.6 millions de personnes atteintes par cette pathologie (diagnostic effectué sur une période inférieur à 5 ans), 14.1 millions de nouveaux cas et 8,2 millions de décès liés à la maladie (environ 13% de mortalité totale). Ce taux est plus élevé dans les pays les moins développés représentant plus de 70% des décès par cancer dans le monde (WHO, 2014).

Le terme « cancer » désigne une prolifération rapide et incontrôlée de cellules anormales (néoplasie) au sein d'un tissu normal. Cette pathologie peut toucher n'importe quelle partie de l'organisme. Les cellules dans ce cas échappent aux mécanismes de régulation et peuvent lorsqu'elles deviennent cancéreuses essaimer à distance dans d'autres organes formant des métastases (WHO, 2014). Ces altérations sont dues à des modifications d'origine génétique ou épigénétique. Contrairement aux mutations génétiques, les changements épigénétiques n'affectent pas la séquence de base de l'ADN et peuvent être réversibles (Peltomaki, 2012).

Les mutations qui peuvent être à l'origine des cancers touchent deux groupes de gènes: les oncogènes et les gènes suppresseurs de tumeurs. L'accumulation de ces mutations peut être facilitée par l'instabilité génomique (Khare and Verma, 2012; Peltomaki, 2012).

Ces modifications proviennent des interactions entre les facteurs génétiques propres à' l'individu et des agents extérieurs pouvant être classés en trois catégories: 1. Agents cancérogènes physiques, comme le rayonnement ultraviolet et les radiations ionisantes; 2. Agents cancérogènes chimiques, comme l'amiante ou les composants de la fumée du tabac; 3. Agents cancérogènes biologiques, comme des infections dues à certains virus, bactéries ou parasites (WHO, 2014).

Une dizaine d'agents infectieux (virus, bactéries et parasites), reconnus oncogènes par l'International Agency for Research on Cancer (IARC) sont capables d'induire la formation des néoplasies chez l'homme. Environ 2 millions (soit 16,1%) des cas de cancer humain survenus en 2008 sont imputables à des infections virales, bactériennes ou parasitaires (Benamrouz et al., 2012).

Parmi les 12,7 millions de nouveau cas recensés en 2008, 1,9 millions des cas ont pour origine une infection par *Helicobacter pylori*, le virus de l'hépatite B, C (HBV et HCV) et le

papillomavirus humain (HPV) et 8000 cas sont dus aux métazoaires *Schistosoma haematobium*, *Opisthorchis viverrini* et *Clonorchis sinensis* (De Martel et al., 2012; WHO, 2014).

En effet, en se basant sur des données cliniques et épidémiologiques, plusieurs articles rapportent une possible relation entre les infections parasitaires et certains cancers. Le protozoaire flagellé *Trichomonas vaginalis* a été décrit comme étant impliqué dans le développement des cancers de la prostate et du col de l'utérus (Benamrouz et al., 2012). De même, plusieurs parasites du genre Apicomplexa ont été associés à des tumeurs. Par exemple, *Toxoplasma gondii* a été associé à des lymphomes, des leucémies, des méningiomes, et des tumeurs oculaires (Khurana, Dubey, and Malla, 2005). D'autre part, l'espèce *Plasmodium falciparum* a été décrite comme un cofacteur dans le développement du lymphome de Burkitt (Khurana, Dubey, and Malla, 2005). Alors que seuls les Apicomplexa de genre *Theileria* et *Cryptosporidium* ont clairement montré expérimentalement une capacité à induire le développement de cancers dans les cellules de l'hôte, des leucocytes et des cellules épithéliales, respectivement (Benamrouz, 2012; Certad et al., 2010a; Certad et al., 2010b; Certad et al., 2007; Dobbelaere and Rottenberg, 2003).

Il a été décrit que pour induire la transformation des cellules de l'hôte, *Theileria* interfère avec les voies de transduction du signal en induisant une inhibition de l'apoptose (Dobbelaere, Fernandez, and Heussler, 2000; Dobbelaere and Rottenberg, 2003).

D'une façon générale, la modulation de l'apoptose a émergé comme un mécanisme important de certains parasites intracellulaires tels que *C. parvum*, *Leishmania* spp, *Trypanosoma cruzi*, *Toxoplasma gondii*, *Plasmodium* spp et *Theileria* spp. L'apoptose des cellules infectées par ces protistes fait partie des mécanismes de défense de l'hôte (Carmen and Sinai, 2007), mais des pathogènes viraux (Roulston, Marcellus, and Branton, 1999), bactériens (Gao and Kwaik, 2000) et parasitaires (Carmen and Sinai, 2007; Heussler, Kuenzi, and Rottenberg, 2001) ont développé des mécanismes d'inhibition de l'apoptoses afin de pouvoir envahir et se multiplier dans des cellules hôtes.

Plus particulièrement, la présence d'une association entre l'infection par *C. parvum* et l'apparition de néoplasies, essentiellement iléocæcales et gastriques a été décrite pour la première fois dans notre laboratoire BDPEE au cours de l'étude histopathologique du tube digestif d'un modèle murin infecté par le parasite, cumulant une immunodépression génétique (SCID) et chimique (traitement avec dexaméthasone). Les processus néoplasiques se sont

développés surtout dans le cæcum mais aussi dans d'autres organes digestifs: estomac, intestin grêle et voies biliaires. Ces lésions ont été observées dès 45 jours post infection (P.I.). Il est à noter que ces mêmes développements néoplasiques ne sont pas observés après infection par *C. muris* et les raisons de ces différences dans l'expression de la maladie restent inconnues (Certad et al., 2012; Certad et al., 2010b; Certad et al., 2007). Une variabilité importante de la pathogénicité en fonction des espèces et variétés infra-spécifiques de *Cryptosporidium* a été précédemment rapportée (Widmer et al., 2012). Selon la chronologie de l'infection et la quantité d'oocystes administrés, les lésions prennent l'allure de polypes, de néoplasies de bas grade ou de haut grade, ou encore d'adénocarcinomes in situ. Une corrélation très significative est décrite entre l'intensité de la cryptosporidiose et la sévérité des lésions observées, suggérant un rôle direct du parasite dans le développement des néoplasies, auxquels il reste systématiquement associé. Ces observations, confortées par les premiers résultats immunohistochimiques obtenus avec le marqueur de prolifération cellulaire Ki67, attestent ainsi de l'induction de tumeurs par le parasite (Certad et al, 2007 ; Certad et al; 2010). Le suivie prolongée des animaux (plus de 60 jours P.I.) a permis de montrer que les lésions deviennent de plus en plus sévères surtout au niveau de la région iléo-caecale et les adénocarcinomes peuvent atteindre la musculeuse et la séreuse. De plus une embolie tumorale et des cholangiocarcinomes ont été décrits après 90 jours P.I. (Figure 1) (Certad et al., 2012).

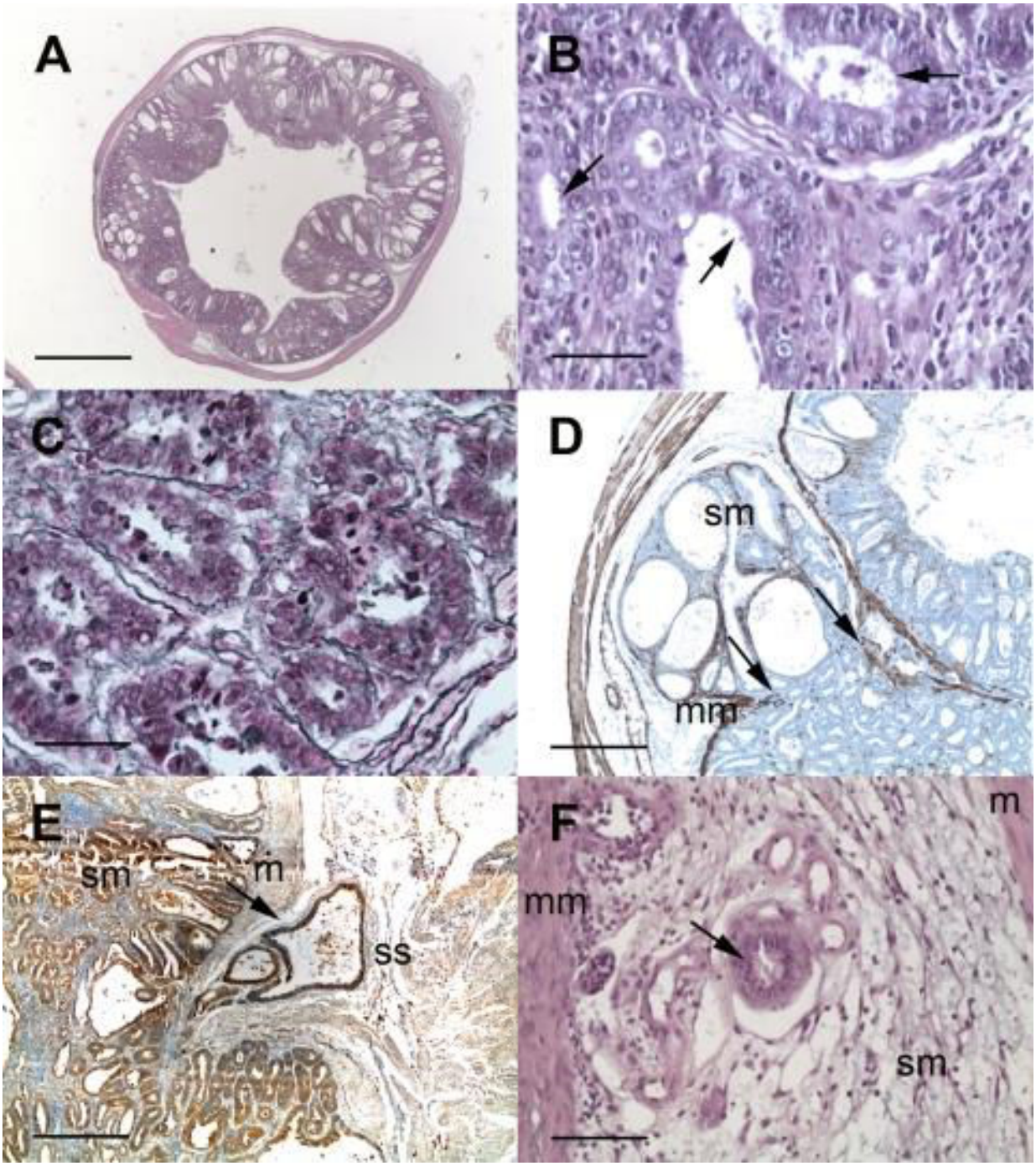

Figure 1 : Observation microscopique du développement des lésions néoplasiques iléo-caecales chez des souris SCID infectées par *C. parvum* d'origine humaine de sous type IIaA15G2R1 en fonction du temps (A→F) de 45 jours P.I. jusqu'à 90 jours P.I.. Coloration réalisée par l'Hématoxyline-Eosine (Certad et al., 2012).

De même, la capacité de *C. parvum* à induire une néoplasie digestive semble toutefois être une propriété intrinsèque de cette espèce et non rattachée à une souche donnée car deux autres souches de *C. parvum* IIaA15G2R1, TUM1 (isolée chez l'animale) et Did (isolée chez l'homme), ont également exprimé un pouvoir carcinogène (Certad et al., 2012; Certad et al., 2010a).

En cohérence avec ces résultats, des données cliniques associant la présence de *Cryptosporidium* et la pathologie cancéreuse ont été évoquées à plusieurs reprises. Un cas a été rapporté chez une patiente espagnole de 64 ans, où les médecins décrivaient une association entre la présence du parasite et un adénocarcinome du colon (Izquierdo et al., 1988). De plus, le CCR a été identifié comme une des "non-AIDS defining malignancies" avec une incidence de plus en plus élevée chez les patients VIH+ par rapport à la population générale (Patel et al., 2008; Yeguez et al., 2003). Ce cancer se développe à un âge plus précoce et d'une façon plus agressive quand les patients sont infectés par le VIH (Yeguez et al., 2003). L'incidence de CCR chez les patients sidéens est 2,3 plus importante que dans la population générale (Patel et al., 2008). Ces patients sidéens sont très fréquemment infectés par *Cryptosporidium* (Certad, 2008).

Plus récemment, deux études polonaises ont montré une prévalence élevée de 18% (2007) et 12.6% (2012) du parasitisme par *Cryptosporidium* chez des patients atteints de CCR en Pologne (Sulzyc-Bielicka et al., 2012; Sulzyc-Bielicka et al., 2007).

De même, une analyse épidémiologique rétrospective réalisée aux Etats Unis a permis de conclure que la cryptosporidiose augmentait significativement le risque de cancer colorectal chez une population de personnes atteintes du SIDA (Shebl, Engels, and Goedert, 2012). Toutefois, dans ce rapport, il est difficile de savoir si *Cryptosporidium* se comporte comme un facteur de carcinogenèse ou simplement comme un agent opportuniste dont le développement a été renforcé par l'immunosuppression de l'hôte.

Dans ce travail de thèse, nous nous intéresserons plus particulièrement au cancer colorectal (CCR) causé par *C. parvum*. Le CCR est une cause importante de morbidité et mortalité dans le monde et est globalement le quatrième cancer le plus fréquent chez l'homme et le troisième chez la femme (WHO, 2014). Il résulte d'une succession d'altérations génétiques qui affectent certains oncogènes, suppresseurs de tumeur ou gènes de stabilité de l'ADN. La séquence de Fearon et Vogelstein représente la séquence de mutations des oncogènes et suppresseurs de tumeur qui conduit à l'altération de voies de signalisation lors de la progression du CCR

(Figure 2) (Knudson, 2001). Les cancers dus à des syndromes héréditaires: FAP (familial adenomatous polyposis), HNPCC (hereditary nonpolyposis colorectal cancer), et d'autres syndromes de polyposes héréditaires (Wiesner, Slavin, and Barnholtz-Sloan, 2009) représente moins de 5% des cancers colorectaux. Alors que les tumeurs dites sporadiques représentent 90% des tumeurs colorectales (Corpet and Pierre, 2005; De Carné Trécesson, 2010). La plupart des CCR résultent d'une progression multi-étapes qui aboutit à un cancer invasif (Benamrouz, 2012).

Les hypothèses émises sont d'une part qu'une association entre la pathologie cancéreuse digestive chez l'homme et le parasitisme par *Cryptosporidium* doit exister. D'autre part, la transformation néoplasique des cellules épithéliales infectées par *C. parvum* pourrait être le résultat d'une modulation des voies de signalisation cellulaire par le parasite. La majorité des cancers colorectaux (CCR) sporadiques humains présentent des mutations de l'APC (Adenomatous Polyposis Coli) ayant pour conséquence la stabilisation de la β-caténine et sa translocation dans le noyau (Buda and Pignatelli, 2004; Giles, van Es, and Clevers, 2003).

L'ensemble de ces données expérimentales chez le modèle murin et clinico-épidémiologiques chez l'homme suggère fortement que le spectre de pathogénicité de *Cryptosporidium*, au moins de l'espèce *C. parvum*, comporte vraisemblablement un pouvoir carcinogène qui s'exprime dans le tractus gastro-intestinal de mammifères immunodéprimés, en incluant probablement l'homme.

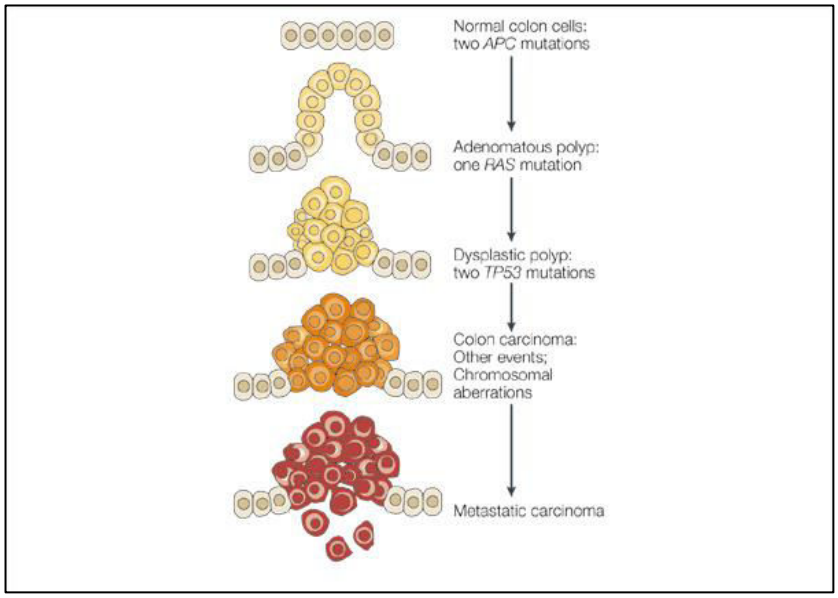

Figure 2. Mécanisme moléculaire du cancer colorectal (CCR). La mise en place et la progression du CCR (progression de l'épithélium sain vers l'adénome puis au cancer) sont des processus multi-étapes. Progressivement les cellules des muqueuses colique et rectale sont le siège de mutations ou de défaillances qui permettent la transition d'une étape à l'autre. La voie Wnt est à l'origine de cette longue marche vers le cancer dans la majorité des CCR. L'APC, gène suppresseur de tumeurs ainsi que le gène Kras, oncogène, sont mutés précocement. Alors que le gène TP53, gène suppresseur de tumeurs, est muté tardivement dans le développement du CCR (Knudson, 2001)

V. *Objectifs et Stratégies*

1. Objectifs :

Ce travail de thèse s'est développé dans le cadre d'un projet collaboratif mené entre le Liban et la France et dont l'objectif général est d'étudier la nature de la diversité génétique de *Cryptosporidium* et l'impact de cette diversité sur la transmission et la pathogénicité du parasite.

Les objectifs spécifiques de cette thèse sont les suivants :

1. Identification des espèces et sous-types de *Cryptosporidium* dans différentes populations et différentes régions géographiques.

2. Etude de l'impact de la diversité génétique de *Cryptosporidium* sur les modalités de transmission et de circulation du parasite.

3. Estimation de l'impact de *Cryptosporidium* en termes de pathogénicité.

2. Stratégies :

Dans le cadre de cette thèse, les axes suivants ont été développés :

1. Le premier axe « **Premières données d'épidémiologie moléculaire et facteurs de risque liés à l'infection par *Cryptosporidium* spp. au Liban**» tente de dresser un premier portrait de l'épidémiologie moléculaire de l'infection par *Cryptosporidium* spp. au Liban, et plus particulièrement dans la région Nord du Liban, en ciblant plusieurs populations humaines.

2. Le deuxième axe « **La prévalence de *Cryptosporidium* spp. dans les échantillons animaux et l'évaluation du pouvoir zoonotique du parasite** » propose une caractérisation de *Cryptosporidium* en termes de circulation aussi bien au Liban qu'en France chez des populations humaines et animales.

3. Le troisième axe « **Etude de la pathogénicité de *Cryptosporidium* spp.** » tente de mettre en évidence une association entre le développement d'un cancer digestif chez l'homme et l'infection par *Cryptosporidium.* Il se base sur les résultats précédemment

obtenus par l'équipe montrant que *C. parvum* est capable d'induire un cancer digestif dans un model murin.

En reprenant ce modèle murin de cryptosporidiose, cet axe tente aussi d'explorer les voies de signalisation pouvant être impliquées lors du processus de cancérogenèse digestive induit par *C. parvum*.

La stratégie méthodologique pour mener à bien ce projet de thèse s'appuie :

- sur une approche d'identification moléculaire pour la détermination de la prévalence et la caractérisation des espèces et sous-types de *Cryptosporidium* parasitant des populations humaines et animales dans différentes régions géographiques.

- sur des enquêtes épidémiologiques menées afin d'identifier de potentiels facteurs de risque liés à cette infection.

- sur une étude clinique mise en place au Liban, un pays à forte incidence parasitaire, afin d'explorer le taux d'infection par *Cryptosporidium spp.* chez des patients porteurs de néoplasies digestives.

- sur des approches moléculaires, immunohistochimiques et microscopiques afin d'explorer les voies de signalisations liées au processus néoplasique induit par le protiste *C. parvum* dans le modèle de souris SCID-Dex et rechercher la présence d'altérations dans des gènes ou dans l'expression de protéines couramment impliqués dans le cas de cancer colorectaux.

VI. *Résultats*

1. Axe 1 : Premières données d'épidémiologie moléculaire et facteurs de risque liés à l'infection par *Cryptosporidium* spp. au Liban

1. Article 1 :

Titre: « Initial data on the molecular epidemiology of cryptosporidiosis in Lebanon ».

Préambule : Cette étude a fait l'objet d'une publication dans le journal PLoS One. 2015 May 7;10(5):e0125129

Résumé :

Les protistes du genre *Cryptosporidium* sont des protozoaires intracellulaires qui infectent le tractus gastro-intestinal ou respiratoire d'un grand nombre de vertébrés, y compris l'homme. La cryptosporidiose, maladie associée à ce parasite, est une infection cosmopolite avec une prévalence très variable selon les pays. Le Liban, à l'instar des pays en développement, est très touché par les parasitoses intestinales. Cependant aucune étude n'a élucidé l'épidémiologie moléculaire de la cryptosporidiose dans ce pays. D'où l'objectif principal de cette étude qui était de déterminer la prévalence de *Cryptosporidium* chez les patients symptomatiques hospitalisés, et d'analyser la diversité génétique des isolats correspondants.

Les selles de 163 patients diarrhéiques âgés de 1 mois à 88 ans ont été collectées dans plusieurs hôpitaux du Nord Liban (Hôpital El Nini de Tripoli ; Hôpital Shifa' de Tripoli ; Hôpital Rahal d'Akkar; et Hôpital Al-Youssef d'Akkar). Un examen microscopique direct ainsi qu'une coloration au Ziehl Neelsen modifiée ont été réalisés sur des selles fraîches afin de rechercher une infection parasitaire. Pour chaque patient, une fiche clinique standardisée a été remplie. Cette fiche nous renseigne sur le type de symptômes gastro-intestinaux, sur la présence d'une éventuelle immunodépression, sur un possible contact avec des animaux, sur des voyages récents à l'étranger, etc. L'ADN total des 163 selles a été extrait à l'aide du Mini kit QIAamp DNA stool (Qiagen®) en suivant les instructions du fabricant. Les ADN obtenus ont été conservés à -20°C jusqu'à leur utilisation. La détection de la présence de *Cryptosporidium* a été réalisée par la mise en évidence d'un fragment d'environ 830 pb du gène codant l'ARNr 18S amplifié par PCR nichée. Les produits de PCR ont été ensuite purifiés puis séquencés. Afin de déterminer les espèces de *Cryptosporidium* responsables de l'infection, les séquences des isolats de *Cryptosporidium* spp. obtenues ont été comparées à

celles d'isolats de *Cryptosporidium* spp. disponibles dans la base de données du National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Afin d'avoir plus d'information sur la transmission de l'infection, le polymorphisme génétique des isolats de *C. parvum* et *C. hominis* a été étudié à l'aide du marquer gp60. Les résultats les plus marquants sont listés ci-dessous:

1. Sur les 163 selles analysées par microscopie, 48,5% (n=79) présentent des infections parasitaires et 6,1% (n=10) ont montré la présence de *Cryptosporidium* (Ziehl Neelsen positif).

2. L'analyse moléculaire a permis de mettre en évidence la présence de *Cryptosporidium* dans 15 échantillons ce qui représente une prévalence de 9.2% de cryptosporidiose chez les patients diarrhéiques au Nord-Liban.

3. Deux espèces ont été identifiées, *C. hominis* (67%) et *C. parvum* (33%).

4. L'analyse du locus gp60 a pu être réalisée sur treize échantillons et la présence d'un sous-type unique IdA19 de *C. hominis* et de deux sous-types de *C. parvum* : IaA15G1R1 (80%) et IIaA15G2R1 (20%) ont été décrits.

En conclusion, nous avons réalisé, à notre connaissance, la première étude d'épidémiologie moléculaire portant sur le parasite *Cryptosporidium* au Liban. Cette étude réalisée dans la région du Nord-Liban a permis de déterminer la prévalence de ce parasite ainsi que la fréquence de différentes espèces et sous-types de *Cryptosporidium*.

<u>Ma contribution dans cette étude a été la suivante:</u>

- Conception de l'étude
- Réalisation des expériences
- Analyse des données
- Rédaction de l'article

Initial data on the molecular epidemiology of cryptosporidiosis in Lebanon

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Abstract

Cryptosporidium spp. represent a major public health problem worldwide and infect the gastrointestinal tract of both immunocompetent and immunocompromised persons. The prevalence of these parasites varies by geographic region, and no data are currently available in Lebanon. To promote an understanding of the epidemiology of cryptosporidiosis in this country, the main aim of this study was to determine the prevalence *Cryptosporidium* in symptomatic hospitalized patients, and to analyze the genetic diversity of the corresponding isolates. Fecal specimens were collected in four hospitals in North Lebanon from 163 patients (77 males and 86 females, ranging in age from 1 to 88 years, with a mean age of 22 years) presenting gastrointestinal disorders during the period July to December 2013. The overall prevalence of *Cryptosporidium* spp. infection obtained by modified Ziehl-Neelsen staining

and/or nested PCR was 11%, and children <5 years old showed a higher rate of *Cryptosporidium* spp. The PCR products of the 15 positive samples were successfully sequenced. Among them, 10 isolates (66.7%) were identified as *C. hominis*, while the remaining 5 (33.3%) were identified as *C. parvum*. After analysis of the gp60 locus, *C. hominis* IdA19, a rare subtype, was found to be predominant. Two *C. parvum* subtypes were found: IIaA15G1R1 and IIaA15G2R1. The molecular characterization of *Cryptosporidium* isolates is an important step in improving our understanding of the epidemiology and transmission of the infection.

Introduction

Cryptosporidium is a protozoan parasite of humans and animals with worldwide distribution. This Apicomplexa is a well-described cause of diarrhea, and is recognized as one of the predominant causes of foodborne and waterborne diseases (Ajjampur et al., 2010; Baldursson and Karanis, 2011). In immunocompetent individuals, cryptosporidiosis may be symptomatic or asymptomatic. In the first case, the most common symptomatology is acute watery diarrhea, lasting up to 2 weeks after exposure to the parasite (7 days on average). Recovery from diarrhea occurs spontaneously in around ten days. Other symptoms, such as abdominal pain, nausea, vomiting, dehydration, asthenia and weight loss, may be present (Current and Garcia, 1991). In immunocompromised individuals, especially in AIDS patients, *Cryptosporidium* oocyst shedding is persistent, and diarrhea becomes chronic and potentially fatal (Chalmers and Davies, 2010).

Due to the ability of *Cryptosporidium* oocysts to resist conventional water treatment methods and cause waterborne outbreaks, the World Health Organization (WHO) has included this fecally/orally transmitted parasite as a reference pathogen in the design and implementation of the WHO guidelines for drinking water quality. Monitoring for oocysts in water is part of the surveillance to support water safety plans (Medema, 2009; WHO, 2011).

The prevalence of cryptosporidiosis reported in developing countries is 2 to 15 times higher than in industrialized countries (Guyot, Sarfati, and Derouin, 2012). This variation can be attributed to better hygiene among inhabitants, and the prevention of contamination of food and water by oocysts in developed countries.

Nevertheless, the transmission routes in the epidemiology of cryptosporidiosis are not yet entirely clarified, largely due to the fact that traditional diagnostic tools do not allow the identification of sources of parasites, and epidemiologic investigations are expensive to

conduct (Xiao, 2010). However, the number of investigations based on the molecular epidemiology of *Cryptosporidium* has increased in the last decade, especially in developing countries, contributing to a better understanding of this public health problem (Jex et al., 2008). In particular, information about the situation and impact of cryptosporidiosis in Lebanon is limited, even though other parasitic infections are prevalent (Hamze et al., 2004).

Cryptosporidiosis occurrence rates vary in Middle Eastern countries. Previous reports based on molecular epidemiology among hospitalized patients have shown differing prevalence as follows: 10% in children and adults in Yemen (Alyousefi et al., 2013), 19% in children in Jordan (Hijjawi et al., 2010) or 49% in children in Egypt (Helmy et al., 2013). *C. parvum* is the predominant species in this geographic region. Despite the high number of subtypes and allele families of *C. parvum* described in these countries, most of the isolates reported belong to two subtype families, IIa and IId (Alyousefi et al., 2013; Hijjawi et al., 2010; Iqbal, Khalid, and Hira, 2011; Nazemalhosseini-Mojarad et al., 2011; Sulaiman et al., 2005). Similarly, several subtypes of *C. hominis* have been reported, with predominance of the subtype families Ib and Id (Hijjawi et al., 2010; Iqbal, Khalid, and Hira, 2011; Nazemalhosseini-Mojarad et al., 2011; Sulaiman et al., 2005). The anthroponotic family of *C. parvum*, IIc, was also found in this region, but in low proportion (Hijjawi et al., 2010; Iqbal, Khalid, and Hira, 2011; Sulaiman et al., 2005). In particular, two previous epidemiologic surveys among Lebanese HIV patients based on microscopy observation described a cryptosporidiosis rate of between 3.1% and 50% (Boujaoude et al., 2000; Naba et al., 2010).

In order to better understand the epidemiology of cryptosporidiosis in Lebanon, the main aim of this study was to determine the prevalence of *Cryptosporidium* in symptomatic hospitalized patients, and to analyze the genetic diversity of the isolates. For the first time at the molecular level, we characterized the species and subtypes of *Cryptosporidium* circulating in Lebanon, a tourist-oriented Middle Eastern country that is a crossroads of the Mediterranean Basin and the Arab hinterland.

Material and Methods:

Ethics Statement

Authorization to conduct this study was obtained from the Lebanese Minister of Public Health (reference number: 4-39716). The institutional directory review boards of Nini and Al-Shifa' Hospitals in Tripoli, and Al-Youssef and Rahal Hospitals in Akkar, also approved the protocol of this project, in agreement with Lebanese legislation. Oral and written

informed consents were obtained from the parents or legal guardians of the children, or directly in the case of adult patients, after a clear explanation of the research objectives. The present study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Sample collection

This study was conducted in the North Governorate of Lebanon (Figure 1). Fecal specimens were collected in four hospitals in North Lebanon (Nini Hospital and Al-Shifa' Hospital in Tripoli; Al-Youssef Hospital and Rahal Hospital in Akkar) from 163 hospitalized patients (77 males and 86 females, ranging in age from 1 to 88 years, with a mean age of 22 years) presenting gastrointestinal disorders during the period July to December 2013 (Table 1). All patients were HIV (Human Immunodeficiency Virus) negative. One fecal sample per patient was collected in a sterile container and transported immediately to the Department of Microbiology of the AZM Center in Tripoli.

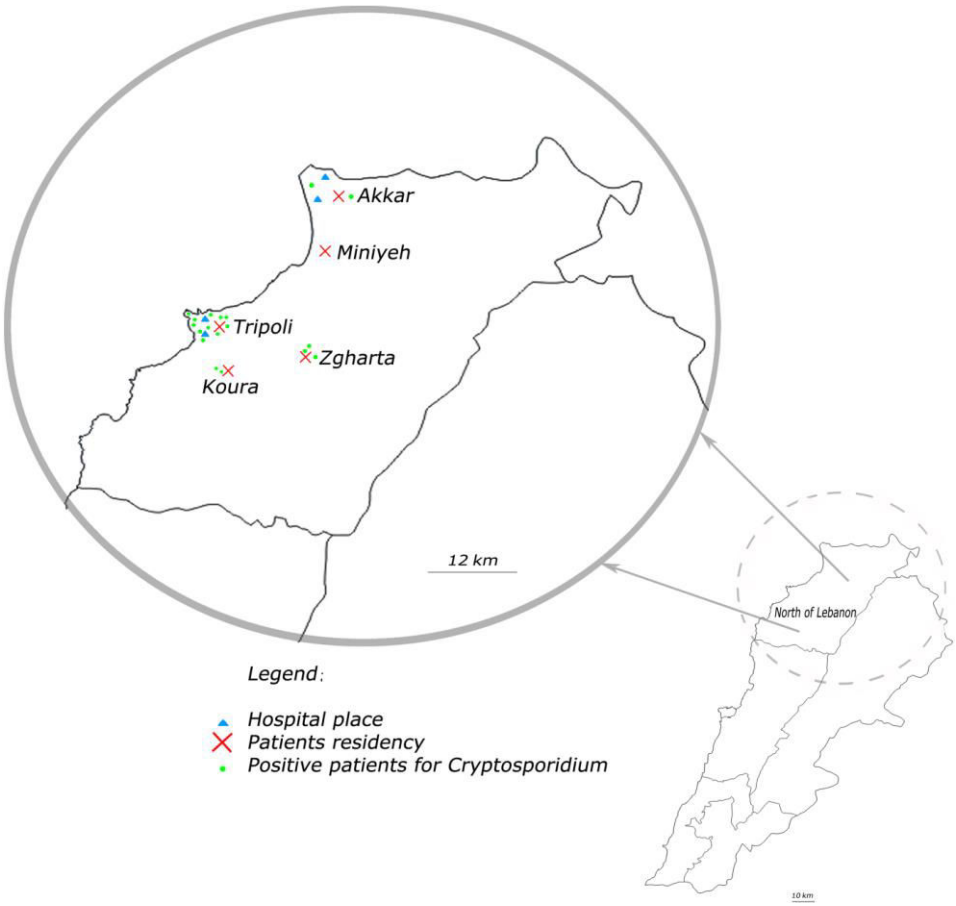

Figure 1. Geographic distribution of cryptosporidiosis cases identified in this study in Lebanon

Microscopic parasitological analyses

All fecal samples were examined macroscopically, and their characteristics, such as color, consistency, presence of blood, or presence of helminths were recorded. These specimens were also examined by direct-light microscopy of wet mounts. For the detection of *Cryptosporidium* spp. oocysts, modified Ziehl-Neelsen (MZN) staining was performed, and the slides were examined at 1000× magnification (Henriksen and Pohlenz, 1981). Two experienced microscopists observed all slides. No information was available on potential viral or bacterial infections in these fecal samples.

DNA extraction, species identification and subtyping

All fecal specimens were used for molecular detection of *Cryptosporidium*. DNA was extracted from approximately 250 µg of fecal samples using the QIAmp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommended procedures. The DNA was eluted in 100 µl of elution buffer (Qiagen) and stored at −20 °C until use. The 18S rRNA nested PCR was performed with primers, as previously described by Xiao *et al*. (Xiao et al., 1999). To test the potential presence of PCR inhibitors in fecal specimens, 0.5 µl of pure DNA of *C. parvum* was added to negative DNA samples and processed by PCR. To identify *Cryptosporidium* spp. molecularly, positive PCR products were purified and sequenced directly by the company Genoscreen (Pasteur Institute, Lille) on both strands using the forward and reverse primers used for the nested (secondary) PCR. The sequences obtained were aligned using the BioEdit v7.0.1 package (http://www.mbio.ncsu.edu:BioEdit/bioedit.html), then compared with sequences of *Cryptosporidium* published on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) using the basic local alignment search tool (BLAST) program. Specimens genotyped as *C. parvum* or *C. hominis* were further subtyped using a second nested PCR, which amplifies a fragment of the 60 kDa glycoprotein (gp60) gene, as described previously (Alves et al., 2003). The amplified DNA fragments were purified, sequenced, and analyzed by alignment of gp60 sequences with reference sequences retrieved from GenBank using the program ClustalX (http://www.clustal.org/). *C. parvum* and *C. hominis* gp60 subtypes were named by counting the number of trinucleotide repeats of TCA (A), TCG (G), and TCT (T) and the ACATCA repeat (R) after the trinucleotide repeats (ANOFEL, 2010). The nucleotide sequences obtained in this study were deposited in GenBank under accession numbers KM215739 to KM215766.

Results

A total of 163 stools from Lebanese hospitalized diarrhea patients were examined. The patients in this study came from different cities in northern Lebanon: 96 from Tripoli, 3 from Menyeh-Doniyeh, 5 from Koura, 10 from Zgharta, and 48 from Akkar. The male/female ratio was 0.9. Of the collected samples, 57.7% (94/163) were from children, and 42.3% (69/163) from adults. Besides diarrhea, patients could present other symptoms, such as abdominal pain (125/163), vomiting (34/163), and fever (21/163). *Cryptosporidium* spp. oocysts were found in 10 out of 163 (6.1%) samples examined by microscopy after MZN staining. After nested PCR analysis, *Cryptosporidium* spp. were detected in 15 samples (9.2%), and a total of 7 specimens out of these 15 samples were simultaneously positive by both methods. For the 3 samples that were positive by MZN staining but negative by nested PCR, the presence of inhibitors was confirmed using a PCR inhibition test. The overall prevalence of *Cryptosporidium* spp. infection obtained by MZN and/or nested PCR was 11% (Table 1), and children <5 years old showed a higher rate of *Cryptosporidium* spp. infection of 44.5% (8/18). The characteristic clinical data of *Cryptosporidium* spp. infected patients is shown in Table 1.

The PCR products of the 15 positive samples were successfully sequenced on both strands. The sequences obtained showed over 99% identity with the reference sequences. Among the isolates, 10 (66.7%) were identified as *C. hominis*, while the remaining 5 (33.3%) were identified as *C. parvum*. *Cryptosporidium* spp. other than *C. parvum* and *C. hominis* were not found.

The partial sequence of the gp60 gene was subsequently obtained for 8 *C. hominis* and 5 *C. parvum* isolates. Sequence analysis of the gp60 gene sequences identified all *C. hominis* isolates as belonging to the IdA19 subtype. Two subtypes of *C. parvum* belonging to the subtype family IIa were identified as follows: IIaA15G1R1 (4/5) and IIaA15G2R1 (1/5) (Table 1). The clinical manifestations associated with *Cryptosporidium* spp. infection are shown in Table 1.

Other intestinal parasites were detected by direct-light microscopy. In total, 79 out of 163 (48.5%) samples were positive for at least one intestinal parasitic infection. The distribution of these infections was as follows: *Blastocystis* spp. with the highest infection rate (19.6%), followed by *Entamoeba histolytica/dispar* (14.1%), *Entamoeba coli* (10.4%), *Giardia duodenalis* (3.7%), *Entamoeba hartmanni* (2.5%), *Taenia* spp. (0.6%), and *Trichomonas*

intestinalis (0.6%). Overall, the prevalence of parasitic infections in males and females was 36.4% and 52.3%, respectively. Co-infection of *Cryptosporidium* spp. with other Protozoa was found in 7 cases (Table 1).

Table 1. Clinical data and *Cryptosporidium* spp. and genotypes among symptomatic patients in Lebanon

Patient identification	Sex	Age (years)	Hospital (residency)	Symptoms	MZN staining	*Cryptosporidium* species (18S rRNA)	Locus gp60	Co-infection
MH1	F	60	Al-Shifa' (Tripoli)	AP/D	+	*C. parvum*	IIaA15G2R1	-
MH2	M	15	Al-Shifa' (Tripoli)	AP/D/F	+	*C. parvum*	IIaA15G1R1	-
MH3	F	76	Nini (Tripoli)	AP/D/V	+	*C. parvum*	IIaA15G1R1	-
MH4	F	32	Nini (Zgharta)	AP/D	+	*C. parvum*	IIaA15G1R1	*E. hartmanni*
MH5	F	1	Nini (Tripoli)	D	-	*C. parvum*	IIaA15G1R1	*E. coli*
MH6	M	62	Nini (Tripoli)	AP/D/V/F	+	*C. hominis*	IdA19	-
MH7	F	7	Nini (Tripoli)	AP/D/V	+	*C. hominis*	IdA19	*E. histolytica*
MH8	M	15	Nini (Koura)	D/V/DH	+	*C. hominis*	IdA19	*E. coli*
MH9	M	2	Nini (Tripoli)	AP/D/V/F/DH	-	*C. hominis*	IdA19	-
MH10	M	1	Nini (Tripoli)	D/V/DH	-	*C. hominis*	IdA19	-
MH11	M	4	Nini (Tripoli)	D/V/F	-	*C. hominis*	IdA19	*E. coli*
MH12	M	2	Al-Youssef (Akkar)	D/V/F/DH	-	*C. hominis*	IdA19	-
MH13	M	1	Al-Youssef (Akkar)	D/V/F	-	*C. hominis*	IdA19	-
MH14	F	58	Al-Shifa' (Tripoli)	AP/D	-	*C. hominis*	-	-
MH15	F	54	Nini (Koura)	AP/D/F	-	*C. hominis*	-	*E. histolytica*
MH16	M	13	Al-Shifa' (Zgharta)	AP/D/V	+	-	-	-
MH17	F	2	Nini (Tripoli)	AP/D	+	-	-	-
MH18	F	1	Nini (Zgharta)	D/V/F	+	-	-	*E. coli*

M: Male, F: Female, AP: Abdominal pain, D: Diarrhea, V: Vomiting, F: Fever, DH: Dehydration, MZN: modified Ziehl-Neelsen

Discussion

The present study represents the first report on molecular data of human cryptosporidiosis in Lebanon. Indeed, only very rare data are available in the literature regarding the situation of cryptosporidiosis in this Middle Eastern country, due to the lack of routine diagnosis of this parasite in medical laboratories. Although only one fecal specimen from each patient was analyzed in this study, thus decreasing the sensitivity of the diagnostic tools, it was shown that *Cryptosporidium* spp. infection was common among hospitalized Lebanese diarrhea patients, with a prevalence of 11%. No gender difference in the prevalence of the parasite was found, and children aged under 5 years were more frequently infected, as described previously (ANOFEL, 2010). Consistently, recent evidential data from the Global Enteric Multicenter Study (GEMS) on the burden and etiology of childhood diarrhea in developing countries has shown that *Cryptosporidium* spp. is nowadays a leading cause of moderate-to-severe diarrhea in children aged less than 2 years (Kotloff et al., 2013).

However, 6 (33%) immunocompetent adults were also infected in our cohort. Although cryptosporidiosis is less common among healthy adults, this observation has been reported previously (ANOFEL, 2010). The hospitalization of these adult patients due to the presence of gastrointestinal symptoms could explain the increase in *Cryptosporidium* detection due to a specific prescription of parasitological methods of diagnosis.

In our study, the frequency of the infection was higher in patients from Tripoli (61%) than from other cities in North Lebanon (Table 1). Globally, based on molecular tools for cryptosporidiosis detection among hospitalized patients, the prevalence of *Cryptosporidium* spp. in Lebanon (11%) was similar to that reported in Yemen (10%) (Alyousefi et al., 2013), but lower than that reported in other neighboring countries, such as Jordan (19%) (Hijjawi et al., 2010) and Egypt (49 %) (Helmy et al., 2013).

In our cohort of hospitalized patients, the molecular characterization of *Cryptosporidium* isolates identified *C. parvum* and *C. hominis*, with the latter being predominant. It is well known that human cryptosporidiosis is mainly caused by these two species, and that the distribution of *C. parvum* and *C. hominis* in humans differs in different geographic regions. In European countries, both *C. parvum* and *C. hominis* are common in humans (Xiao, 2010). In the Middle East, however, *C. parvum* is the dominant species in the human population (Xiao, 2010). In the rest of the world, especially in developing countries, *C. hominis* is usually the predominant species in humans (Xiao, 2010), and the predominance of this

species is related to anthroponotic transmission. Anthroponotic transmission of *Cryptosporidium* spp. has thus been described in several tropical countries, such as Peru, Thailand, Malawi, Uganda, Kenya, and South Africa (Xiao, 2010).

In this study, all *C. hominis* isolates belonged to the Id subtype family, and were identified as belonging to the IdA19 subtype. Although the Id family is commonly reported around the world (Xiao, 2010), the IdA19 subtype is less common. This subtype was previously described in Northern Ontario in Canada (Trotz-Williams et al., 2006), and in hospitalized children in China (Feng et al., 2012). In addition, patients with the IdA19 subtype presented severe symptoms (4 cases of dehydration and 7 cases of vomiting) in our report. Consistently, Iqbal *et al.* showed that the Id subtype family was associated with severe diarrhea lasting more than 6 days, and also associated with other clinical manifestations, such as fever and dehydration in Kuwaiti children (Iqbal, Khalid, and Hira, 2011). This finding was also supported by an earlier study showing that the *C. hominis* Id subtype family was more virulent than other subtype families in Peruvian AIDS patients (Cama et al., 2007). However, in the Chinese study mentioned above, IdA19 was not significantly associated with diarrhea (Feng et al., 2012).

On the other hand, two subtypes of *C. parvum* belonging to the IIa subtype family were identified in our study: IIaA15G1R1 (4/5) and IIaA15G2R1 (1/5). IIaA15G2R1 is the major subtype of *C. parvum* reported around the world, including in the Middle East and the Mediterranean Basin (Xiao, 2010). The *C. parvum* IIa subtype family, of which IIaA15G1R1 and IIaA15G2R1 are representatives, exhibits extensive genetic diversity and is responsible for the majority of cryptosporidiosis outbreaks due to *C. parvum* (Xiao, 2010). Moreover, this subtype family is dominant in the human population in the Middle East, with the exception of Jordan and Iran (Alyousefi et al., 2013; Hijjawi et al., 2010; Iqbal, Khalid, and Hira, 2011; Nazemalhosseini-Mojarad et al., 2011), and has been identified in both humans and animals. The IIc subtype family, associated with anthroponotic transmission, has been described in other Middle Eastern countries, but was not identified in our study (Iqbal, Khalid, and Hira, 2011; Sulaiman et al., 2005).

Interestingly, the co-infection of *Cryptosporidium* spp. with pathogenic and nonpathogenic amoebas was found in several patients in our Lebanese cohort. Since amoebas are also transmitted by the fecal-oral route, these co-infections could indicate a common route of transmission for these parasites, probably due to contaminated food and water.

In conclusion, to our knowledge, this study is the first investigation regarding the molecular epidemiology of *Cryptosporidium* spp. in Lebanon. Our data indicates that the prevalence of cryptosporidiosis is relatively frequent among hospitalized diarrhea patients, in particular in children. Due to the limited number of isolates analyzed in this study, the epidemiologic significance of these results remains to be confirmed. For a better understanding of the circulation of this parasite and its transmission risk factors, further studies including a large number of human, animal, and environmental samples are needed.

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2. Article 2 :

Titre: «Prevalence and Risk Factors for Intestinal Protozoan Infections with *Cryptosporidium*, *Giardia*, *Blastocystis* and *Dientamoeba* among Schoolchildren in Tripoli, Lebanon ».

Préambule : Cette étude a fait l'objet d'un article soumis au journal « Plos Neglected Tropical Diseases ».

Résumé :

Les infections parasitaires sont un problème de santé publique majeur dans le monde. Leur fréquence varie selon différents facteurs : localisation géographique, saisonnalité, population cible, méthodes de diagnostic employées, etc. Au Liban, à l'instar de la plupart des pays en développement, les infections parasitaires intestinales sont à l'origine d'une morbidité significativement élevée, en particulier chez les enfants. Cependant, peu d'informations sont disponibles concernant la prévalence et les facteurs de risque liés à ces maladies dans ce pays. Or, ces parasites cosmopolites, en particulier *Cryptosporidium* et *Giardia*, sont considérés comme des causes de gastroentérites et de nouveaux cas sont recensés chaque année dans le monde. L'identification moléculaire des espèces et sous-types ainsi que la recherche des facteurs de risque liés à ces infections parasitaires chez l'homme sont indispensables pour mieux comprendre l'épidémiologie, et notamment les modes de transmission de ces maladies.

Les selles de 249 enfants âgés de 3 à 16 ans et fréquentant deux écoles de Tripoli (Nord-Liban) de niveau sociaux économiques différents ont été récoltées. Un questionnaire résumant certaines informations comme le statut socio-économique et démographique, les habitudes comportementales, la présence de troubles digestifs, les antécédents d'hospitalisation et de traitement, le contact éventuel avec des animaux et/ou des voyages récents à l'étranger, a été complété pour chaque enfant. Dans un premier temps, la prévalence des infections parasitaires impliquant *Blastocystis* sp, *Giardia duodenalis*, *Cryptosporidium* spp, *Entamoeba histolytica/dispar*, *Entamoeba coli*, *Dientamoeba fragilis*, *Taenia* sp, *Ascaris*

lumbricoides et *Hymenolepis nana* a été déterminée par examen microscopique direct des selles après concentration par le formol/éther puis par coloration au Ziehl Neelsen modifiée.

Ensuite, l'ADN total a été extrait à partir des selles en utilisant le QIAamp DNA Stool Mini Kit (QIAGEN). La détection de *Cryptosporidium*, *Giardia*, *Blastocystis* et *Dientamoeba* dans tous les échantillons a été réalisée par biologie moléculaire. Les produits de PCR de *Cryptosporidium* et *Blastocystis* ont été séquencés afin de déterminer l'espèce de *Cryptosporidium* et du sous-type de *Blastocystis* présents dans les échantillons. Le polymorphisme génétique des isolats de *C. parvum* et *C. hominis* a été étudié à l'aide du marqueur gp60. Les résultats sont les suivants :

1. Au total, 88% des enfants fréquentant l'école de bas niveau socio-économique sont infectés par au moins un parasite intestinal et 80% des enfants fréquentant l'école de haut niveau socio-économique.

2. En analysant toutes les données cliniques et épidémiologiques, on note que *Blastocystis* sp. (63%) et *Dientamoeba fragilis* (61%) sont les parasites intestinaux les plus fréquemment retrouvés chez ces enfants quel que soit l'école fréquentée même si sa prévalence est légèrement plus faible dans l'école de plus haut niveau socio-économique. C'est d'ailleurs le cas pour toutes les autres infections parasitaires à l'exception de *Giardia duodenalis* et *Entamoeba histolytica/dispar*. Ces deux derniers parasites sont significativement plus fréquents chez les enfants fréquentant l'école de plus bas niveau socio-économique ($P < 0.05$).

3. L'infection des parents semble avoir un rôle majeur dans la transmission des parasites aux enfants. La consommation d'une eau non-traitée ainsi que de fruits et légumes crus sont des facteurs de risque pour l'infection par *Giardia duodenalis*. De plus, la consommation des aliments à l'extérieur constitue un facteur de risque pour l'infection par *Cryptosporidium* spp.

4. L'infection par *Cryptosporidium* spp., *Giardia duodenalis* et *Entamoeba histolytica/dispar* est accompagnée de symptômes digestifs, notamment des douleurs abdominales et des diarrhées.

5. Une association a été montrée entre la présence de *Dientamoeba fragilis* et celle de *Blastocystis* sp. ($P<0.0001$) et entre la présence de *Dientamoeba fragilis* et celle d'*Entamoeba coli* ($P=0.04$).

6. Nos résultats de séquençage ont permis d'identifier trois sous-types de *Blastocystis* : ST1, ST2 et ST3. Pour *Cryptosporidium* deux espèces ont été identifiées, *C. hominis* (77%) et *C. parvum* (23%). L'analyse des isolats de *C. hominis* et *C. parvum* par le marqueur gp60 a montré la présence des sous-types IbA10G2 (80%) et IaA18R3 (20%) de *C. hominis* et IaA15G1R1 (100%) de *C. parvum*.

En conclusion, cette étude a généré les premiers résultats d'épidémiologie moléculaire de plusieurs parasites intestinaux au Liban. De plus, plusieurs facteurs de risque liés à l'infection parasitaire ont été détectés et une association très significative entre les infections par les deux parasites *Dientamoeba fragilis* et *Blastocystis* sp. a été confirmée. Nos résultats de génotypage viennent en cohérence avec nos études précédentes au Liban montrant que le ST3 et *C. hominis* sont prédominants chez les enfants. Ces résultats accentuent la prise de conscience de la nécessité de la mise en place de mesures pour prévenir les infections parasitaires liées au péril fécal, notamment chez les enfants au Liban.

Ma contribution dans cette étude a été la suivante:

- Conception de l'étude
- Réalisation des expériences
- Analyse des données
- Rédaction de l'article

Prevalence and Risk Factors for Intestinal Protozoan Infections with *Cryptosporidium, Giardia*, *Blastocystis* and *Dientamoeba* among Schoolchildren in Tripoli, Lebanon

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Abstract

Background: Intestinal protozoa infections are confirmed as major causes of diarrhea, particularly in children, representing a significant but often neglected threat to public health. No recent data was available in Lebanon concerning the molecular epidemiology of protozoan infections in children, a vulnerable population at high risk of infection.

Methodology and Principal Findings: In order to increase our understanding of the epidemiology of intestinal pathogenic protozoa, a cross-sectional study was conducted. After obtaining informed consent from the parents or legal guardians, stool samples were collected in January 2013 from 249 children in 2 schools in Tripoli, Lebanon. Information obtained through a standard questionnaire included demographic characteristics, current symptoms, socioeconomic status, source of drinking water, and personal hygiene habits. After fecal examination by both microscopy and molecular tools, the overall prevalence of parasitic infection was recorded as 85%. *Blastocystis* sp. presented the highest infection rate (63%), followed by *Dientamoeba fragilis* (60.6%), *Giardia duodenalis* (28.5%) and *Cryptosporidium* spp. (10.4%). PCR was also performed to identify *Blastocystis* subtypes and *Cryptosporidium* species. In addition, the analysis of the highly polymorphic 60-kDa glycoprotein (gp60) allowed the subtyping of *C. hominis* and *C. parvum* isolates. Statistical analysis using a logistic regression model showed that contact with family members presenting gastrointestinal disorders was the primary risk factor for transmission of these protozoa.

Conclusions: This is the first study performed in Lebanon reporting the prevalence, clinical and molecular epidemiological data associated with intestinal protozoan infections among schoolchildren in Tripoli. A high prevalence of protozoan parasites was found, where *Blastocystis* sp. was the most predominant protozoan. Although only 50% of children reported digestive symptoms, asymptomatic infection was observed, and these children may act as unidentified carriers. This survey provides necessary information to the public health authorities for designing prevention and control strategies to reduce the burden of these protozoan infections, especially in children.

Summary

Intestinal parasites can infect the gastrointestinal tract of humans. Means of exposure include ingestion of contaminated fruits and vegetables, consumption of infected water and personal contact. Protozoa are considered one of the major groups of parasites. Children are particularly susceptible to infection by these microorganisms, and when they are infected, diarrhea can be the main clinical manifestation. In developing countries people are at particular risk of infection. However, intestinal parasites, and in particular protozoan are taken into account only in few epidemiologic studies. In this way we carried out an investigation to determine the prevalence, risk factors and epidemiological information associated with 4 intestinal protozoan infections: *Cryptosporidium*, *Giardia*, *Blastocystis* and *Dientamoeba,* among children attending two schools of Tripoli, Lebanon. A high prevalence of protozoan parasites was found. Although only 50% of children reported digestive symptoms, asymptomatic infection was observed very often suggesting that these children may act as unknown carriers. In addition, we found that personal contact plays an important role as risk factor associated to protozoan infection. The results of this study will help to make the public health authorities aware of the importance of parasitic infections in this population. In a near future they could establish strategies for disease control and prevention.

Introduction

Parasitic infections, and in particular those caused by protozoa, are a major public health problem worldwide. They are among the most widespread human infections in developing countries, with children being the most vulnerable population (Harhay, Horton, and Olliaro, 2010).

In particular, intestinal protozoan infections, such as those caused by *Cryptosporidium* and *Giardia*, are both confirmed causes of diarrhea in children. Recent data from the Global Enteric Multicenter Study (GEMS) on the burden and etiology of childhood diarrhea in developing countries have shown that the apicomplexan protists *Cryptosporidium* spp. are nowadays one of the leading cause of moderate-to-severe diarrhea in children aged under 2 years (Kotloff et al., 2013; Striepen, 2013). In addition, *Giardia duodenalis* infects approximately 200 million individuals worldwide, and is particularly common among schoolchildren and in day-care centers (Heresi, Murphy, and Cleary, 2000). In children under 5 years, *G. duodenalis* infection may produce severe acute diarrhea. Several studies have

suggested that long-term growth retardation can be a consequence of chronic giardiasis (Maikai et al., 2012).

Other parasites, such as *Blastocystis* sp. and *Dientamoeba fragilis*, are cosmopolitan protozoa found in the gastrointestinal tract of humans. Nevertheless, the exact contribution of *Blastocystis* sp. and *D. fragilis* to pathogenicity has been controversial. The prevalence of *Blastocystis* sp. in humans varies, from 0.5%–24% in industrialized countries and to 30%–76% in developing countries (Wawrzyniak et al., 2013). Recently, a *Blastocystis* sp. prevalence of 100% was found in a Senegalese population of children, being the highest prevalence ever reported worldwide for this parasite (El Safadi et al., 2014). All cases were caused by subtypes (STs) 1, 2, 3 and 4, with a predominance of ST3. The prevalence of *D. fragilis* ranges from 1% to 52%, according to different geographic regions (Barratt et al., 2011).

Recent studies support the pathogenic nature of both parasites. More than half of the children infected by *Blastocystis* sp. in Senegal presented various gastrointestinal disorders (El Safadi et al., 2014), and it is now accepted that the classic clinical features of infection with this parasite include gastrointestinal symptoms such as nausea, anorexia, flatulence, and acute or chronic diarrhea (Clark et al., 2013). An association of *Blastocystis* sp. with irritable bowel syndrome (IBS) (Poirier et al., 2012) and extraintestinal manifestations such as urticaria has also been suggested (Verma and Delfanian, 2013). Moreover, an invasive and inflammatory potential of the parasite has been reported (Frealle et al., 2015).

Regarding *D. fragilis*, infection can be acute or chronic, and symptomatic patients exhibit abdominal pain, persistent diarrhea, loss of appetite, weight loss and flatulence, as well as IBS-like symptoms (Fletcher et al., 2014). Symptoms are observed in 20–58% of infected cases. It has been proposed that *D. fragilis* could be a heterogeneous species, with variants having similar morphology but differing in virulence (Vandenberg et al., 2006).

In Lebanon, as in other developing countries, intestinal parasitic infections remain responsible for significant morbidity (Hamze et al., 2004; Hamze, Naja, and Mallat, 2008). Nevertheless, concerning *Blastocystis* sp. and *Cryptosporidium* spp., only preliminary data is available that reports a prevalence of 19% and 11%, respectively, among hospitalized patients (El Safadi et al., 2013; Osman et al., 2015); and regarding *Dientamoeba* and *Giardia* infections, no epidemiological data is available. In addition, little information is available in this country on the potential risk factors associated with these protozoan infections in children.

Therefore, the aim of this study was to identify potential risk factors for transmission and acquire molecular epidemiological data on the prevalence and genetic diversity of *Cryptosporidium*, *Giardia*, *Blastocystis* and *Dientamoeba* in a population of children attending two schools of different socio-economical level in Tripoli, Lebanon.

Materials and methods

Ethics Statement

Authorization to conduct this study was obtained from the Lebanese Minister of Public Health (reference number: 4-39716). Written informed consents were obtained from the parents or legal guardians of the children, after a clear explanation of the research objectives. This study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Questionnaire survey

A standard questionnaire was completed by interviewing the child's parents or legal guardians, who had given informed consent, in order to obtain a socioeconomic and demographic description such as age, gender, education, residence, occupation and estimated monthly income of the parents, behavioral habits (intake of fruits, vegetables and fast food), health conditions, presence of symptoms (i.e. abdominal pain, diarrhea, vomiting, fever, nausea, headache and discomfort), family members with gastrointestinal disorders, history of previous hospitalizations and medical treatments. Environmental conditions, such as type of water supply, sewage disposal system and presence of domestic animals, were also investigated.

Study population and collection of samples

This cross-sectional study was conducted in Tripoli (Latitude: 34° 26' 12 N, Longitude: 35° 50' 58 E), the largest city in northern Lebanon, and the second largest city in the country in terms of demographic and economic importance. The city, situated 85 kilometers (53 miles) north of the capital Beirut, has a Mediterranean climate with mild winters and moderately hot summers. Tripoli's population has been estimated to be 500,000 at people. Fecal samples were collected at 2 nearly schools of different socio-economical status in the city (Al Zahra' School and Jil Alwa'ed School) (Figure 1) from two hundred and forty-nine children (149 boys and 100 girls aged between 3 and 16 years) in January 2013. The sample size

corresponds to the total number of samples that we could collect for logistical reasons during a specific period of time. The participants were categorized into three groups according to age: under 5 years, between 5 and 9 years and over 9 years, and into two groups according to socioeconomic status: low socioeconomic status (LSES) and high socioeconomic status (HSES). The measure of SES was based on the income, education and occupation of the parents. One fresh fecal sample per child was collected in a sterile container and transported immediately to the Department of Microbiology of the AZM Center in Tripoli.

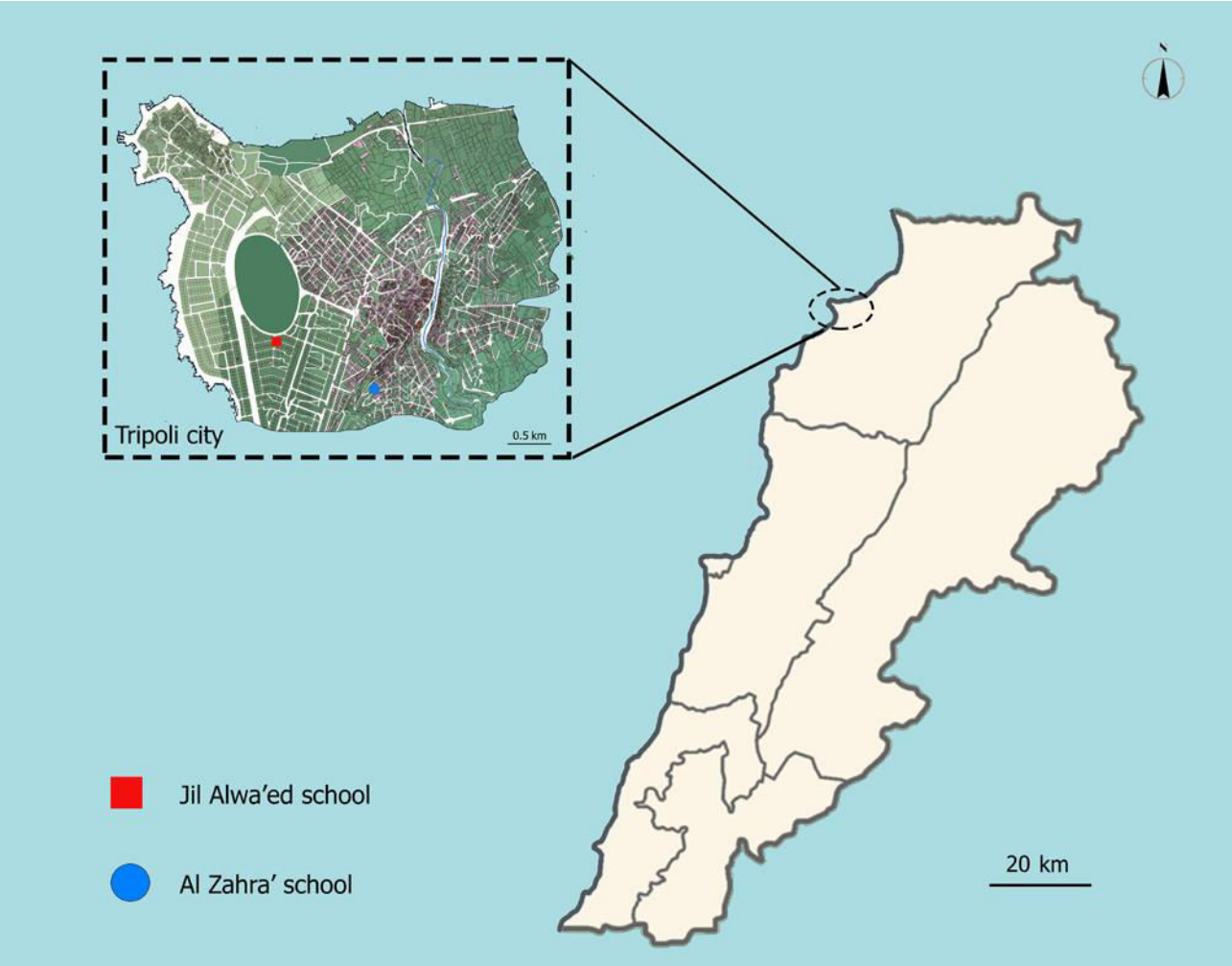

Figure 1. Map of Tripoli, showing the location of Al Zahra' and Jil Alwa'ed schools

Parasitological analyses

All fecal samples were examined macroscopically, and their characteristics such as color, consistency, presence of blood, and presence of helminths were recorded. These specimens were also examined by direct-light microscopy (DLM) of wet mounts. For the detection of

Cryptosporidium spp. oocysts, modified Ziehl-Neelsen (MZN) staining was performed (Henriksen and Pohlenz, 1981), and the slides were examined at 1,000× magnification. For quality control, all examinations were repeated twice by two experienced microscopists. No information was available about potential viral or bacterial infections in these fecal samples.

DNA extraction, species identification and subtyping

All fecal specimens were used for molecular detection of *Blastocystis*, *Cryptosporidium*, *Dientamoeba and Giardia.* DNA was extracted from approximately 250 mg of fecal samples using the QIAmp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommended procedures. The DNA was eluted in 100 µl of elution buffer (Qiagen) and stored at −20 °C until use. The 18S rRNA detection was performed by nested PCR for *Cryptosporidium* spp. (Xiao et al., 1999) and by real-time PCR for *Blastocystis* sp. (Poirier et al., 2011), *Dientamoeba* (Stark et al., 2006) and *Giardia* (Verweij et al., 2003), as previously described. To further identify *Blastocystis* sp. STs and *Cryptosporidium* spp., positive PCR products were purified and sequenced directly on both strands. The sequences obtained were aligned using the BioEdit v7.0.1 package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), then compared with all SSU rRNA gene sequences of these parasites available from the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/), using the basic local alignment search tool (BLAST). *Blastocystis* sp. STs were identified by determining the exact match or closest similarity against all known STs, according to the updated classification of Alfellani *et al* (Alfellani et al., 2013b). Specimens genotyped as *C. parvum* or *C. hominis* were further subtyped using a nested PCR in order to amplify a fragment of the 60 kDa glycoprotein (gp60) gene, as described previously (Alves et al., 2003). The amplified DNA fragments were purified, sequenced and analyzed by alignment of gp60 sequences with reference sequences retrieved from GenBank using the ClustalX program (http://www.clustal.org/). *C. parvum* and *C. hominis* gp60 subtypes were named by counting the number of trinucleotide repeats of TCA (A), TCG (G), and TCT (T), and the ACATCA repeat (R) after the trinucleotide repeats (Alyousefi et al., 2013).

Statistical analyses

Statistical analysis was performed using Stata software, version 13 (StataCorp, College Station, TX, US). The tests were two-sided, with a type I error set at α=0.05. Quantitative data were presented as the mean ± standard deviation or the median [interquartile range]. The

categorical data were presented as frequency and associated proportions. The differences across groups were compared using (1) Student's t or Mann-Whitney U test when conditions of t-test were not met for continuous variables (assumption of normality studied using Shapiro-Wilk test and homoscedasticity by Fisher-Snedecor test), and (2) Chi-squared test or Fisher's exact test for categorical parameters. Logistic regression models were performed to calculate odds ratios (OR) and 95% confidence interval considering parasite infections as the main outcome. Analyses were based on parasite detection using molecular tools.

Results

Prevalence of protozoan infections

A total of 249 schoolchildren (149 males, 100 females) were included in this study. Among them, 157 belonged to the LSES group (mostly children from the Al-Zahra' school) and the remaining 92 to the HSES group (mostly children from the Jil Alwa'ed school). The age of the participants was between 3 and 16 years (mean age: 10.3 ± 2.7) (Table 1).

Overall, based on PCR and light microscopy examination, 85% (212/249) of children were found to be positive for at least one intestinal parasitic infection. Out of a total of 212 infected schoolchildren, the distribution of parasitic infections in males and females was 61% (129/212) and 39% (83/212), respectively. When socioeconomic status was considered, the prevalence was as follows: 65% (138/212) of children in the LSES group and 35% (74/212) in the HSES group. No significant statistical differences regarding parasitic infections related to gender or socioeconomic status were observed. Demographic characteristics of the study population are shown in Table 1.

Table 1. Demographic characteristics of the study population

	Non-infected children (N=37)	Infected children (N=212)
Age (median)	8.48 ± 0.50	9.5 ± 0.21
Gender		
Male	20	129
Female	17	83
Children in the LSES group	19	138
Children in the HSES group	18	74

LSES: low socioeconomic status, HSES: high socioeconomic status

After molecular analysis of the samples, *Blastocystis* sp. had the highest infection rate (63%), followed by *D. fragilis* (60.6%), *G. duodenalis* (28.5%) and *Cryptosporidium* spp. (10.4%). As expected, the prevalence of these protozoa was lower in microscopic examination of wet mounts (51.6%, 14.4%, 5.6% and 0%, respectively). Other intestinal parasites were also detected by DLM, as follows: *Entamoeba histolytica/dispar* (5.6%), *Entamoeba coli* (2.4%), *Ascaris lumbricoides* (0.4%), and *Hymenolepis nana* (0.4%) (Figure 2).

Mixed infections with two parasites were found in 35.7% of children (89/249). The most common dual infection was with *Blastocystis* sp. and *D. fragilis*, with a prevalence of 68.5% (61/89). In addition, 11.6% (29/249) of children exhibited triple parasitic infections with *Blastocystis* sp., *D. fragilis* and *G. duodenalis*. Other cases of mixed infections are shown in Figure 2.

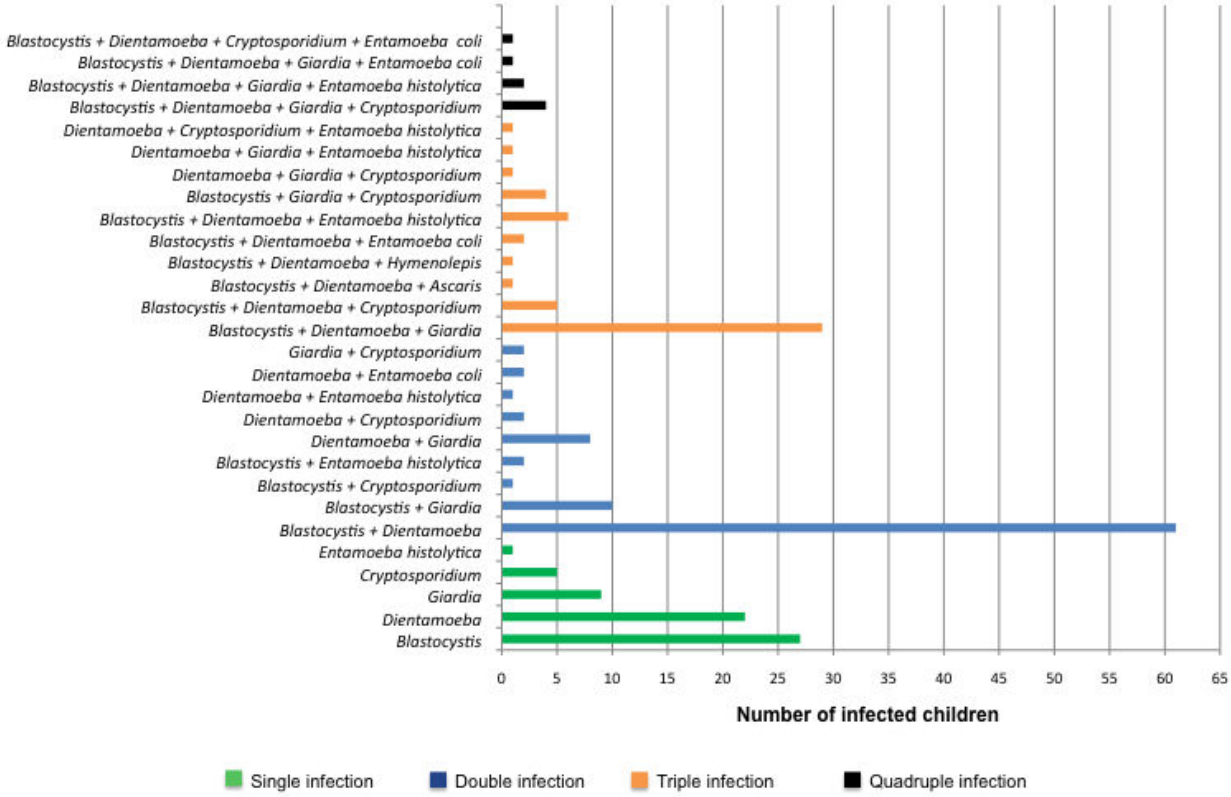

Figure 2. Distribution of single and mixed parasitic infections in schoolchildren in Tripoli

Clinical manifestations and risk factors for transmission

In total, 125 out of 249 children had symptoms at the time of the survey. Among parasitized children, gastrointestinal symptoms were common (55%). Abdominal pain, diarrhea, vomiting and fever were reported in 51% (108/212), 28 % (60/212), 11% (23/212) and 6% (12/212) of children, respectively.

A logistic regression model was created to identify the risk factors for transmission of intestinal parasitic infections. Overall, the presence of abdominal pain (OR: 5.4, CI: 2.1-13.4, P<0.001) and diarrhea (OR: 4.5, CI: 1.3-15.1, P: 0.009), and having parents with gastrointestinal symptoms (OR: 9.6, CI: 2.2-40.9, P<0.001) were significantly predictive of the risk of intestinal parasitic infections in children.

After individual analysis of each protozoan infection, we found that 51.6% (81/157) of *Blastocystis* sp.-infected children reported abdominal pain. Other symptoms included diarrhea, vomiting and fever in 28.7% (45/157), 10.8% (17/157), and 3.8% (6/157) of cases, respectively. Of the total of 157 *Blastocystis* sp.-infected children, 71 were asymptomatic. Univariate logistic regression analysis showed the presence of abdominal pain (OR: 1.9, CI: 1.1-3.2, P: 0.02) and contact with parents having gastrointestinal symptoms (OR: 1.9, CI: 1.0-3.4, P: 0.03) to be the main factors significantly associated with *Blastocystis* sp. infection. Animal contact (OR: 0.3, CI: 0.1 – 0.8, P: 0.1) and eating outside the home (OR: 0.5, CI: 0.3 – 0.9, P: 0.02) seem to represent protective factors against *Blastocystis* sp. infection (Table 2).

Table 2. Distribution of protozoan infections among schoolchildren in Tripoli according to risk factors

		***Blastocystis* sp.**		***Dientamoeba fragilis***		***Giardia duodenalis***		***Cryptosporidium* spp.**	
Risk factor		Prevalence *% (N)	*P*-value OR (IC95%)	Prevalence *% (N)	*P*-value OR (IC95%)	Prevalence *% (N)	*P*-value OR (IC95%)	Prevalence *% (N)	*P*-value OR (IC95%)
Age	**< 5 years**	61.5% (8/13)	1.0 0.93 [0.3 – 2.9]	53.8% (7/13)	0.77 0.74 [0.2 – 2.3]	23.1% (3/13)	0.76 0.74 [0.2 – 2.8]	38.4% (5/13)	**0.006 6.4 [1.9 – 21.3]**
	≥ 5 years	63.1% (149/236)		61% (144/236)		28.8% (68/236)		8.9% (21/236)	
Sex	**Male**	66.4% (99/149)	0.18 1.4 [0.9 – 2.4]	63.1% (94/149)	0.34 1.3 [0.8 – 2.2]	31.5% (47/149)	0.20 1.5 [0.8 – 2.6]	10.7% (16/149)	0.85 1.1 [0.5 – 2.5]
	Female	58% (58/100)		57% (57/100)		24% (24/100)		10% (10/100)	
Socio- economic status	**Low**	66.2% (104/157)	0.18 1.4 [0.8 – 2.4]	63.7% (100/157)	0.23 1.4 [0.8 – 2.4]	36.3% (57/157)	**<0.001 3.2 [1.6 – 6.1]**	10.8% (17/157)	0.83 1.1 [0.5 – 2.6]
	High	57.6% (53/92)		55.4% (51/92)		15.2% (14/92)		9.8% (9/92)	

Contact with animals	**Yes**	36.8% (7/19)	**0.01** **0.3** **[0.1 – 0.8]**	57.9% (11/19)	0.80 0.9 [0.3 – 2.3]	15.8% (3/19)	0.20 0.4 [0.1 – 1.6]	15.8% (3/19)	0.43 1.7 [0.5 – 6.2]
	No	65.2% (150/230)		60.9% (140/230)		29.6% (68/230)		10% (23/230)	
Raw fruit and vegetable consumption	**Yes**	64.6% (126/195)	0.33 1.4 [0.7 – 2.5]	62.1% (121/195)	0.39 1.3 [0.7 – 2.4]	32.3% (63/195)	**0.01** **2.7 [1.2 – 6.2]**	9.7% (19/195)	0.49 0.7 [0.3 – 1.8]
	No	57.4% (31/54)		55.6% (30/54)		14.8% (8/54)		13% (7/54)	
Treated water supply in household	**Yes**	58.3% (35/60)	0.38 0.8 [0.4 – 1.4]	56.7% (34/60)	0.47 0.8 [0.4 – 1.5]	13.3% (8/60)	**0.003** **0.3** **[0.1 – 0.7]**	13.3% (8/60)	0.40 1.5 [0.6 – 3.6]
	No	64.6% (122/189)		61.9% (117/189)		33.3% (63/189)		9.5% (18/189)	
Meals outside the home	**Yes**	50% (28/56)	**0.02** **0.5** **[0.3 – 0.9]**	50% (28/56)	0.06 0.6 [0.3 – 1.0]	12.5% (7/56)	**0.003** **0.3** **[0.1 – 0.7]**	17.9% (10/56)	**0.04** **2.4** **[1.1 – 5.6]**
	No	66.8% (129/193)		63.7% (123/193)		33.2% (64/193)		8.3% (16/193)	
Parents with gastrointestinal symptoms	**Yes**	72.8% (56/77)	**0.03** **1.9** **[1.0 – 3.4]**	72.8% (56/77)	**0.01** **2.2** **[1.2 – 3.9]**	51.9% (40/77)	**<0.001** **4.9** **[2.7 – 8.9]**	14.3% (11/77)	0.18 1.7 [0.8 – 4.0]
	No	58.7% (101/172)		55.2% (95/172)		18% (31/172)		8.7% (15/172)	

Digestive symptoms	**Yes**	68.8% (86/125)	0.06 1.6 [0.9 – 2.7]	64% (80/125)	0.27 1.3 [0.8 – 2.2]	42.4% (53/125)	**<0.001** 4.3 [2.8 – 8.0]	15.2% (19/125)	**0.01 3.0 [1.2 – 7.4]**
	No	57.3% (71/124)		57.3% (71/124)		14.5% (18/124)		5.6% (7/124)	
Abdominal pain	**Yes**	71.1% (81/114)	**0.02 1.9 [1.1 – 3.2]**	65.8% (75/114)	0.13 1.5 [0.9 – 2.5]	44.7% (51/114)	**<0.001 4.7 [2.6 – 8.5]**	13.2% (15/114)	0.20 1.7 [0.8 – 3.9]
	No	56.3% (76/135)		56.3% (76/135)		14.8% (20/135)		8.1% (11/135)	
Diarrhea	**Yes**	71.4% (45/63)	0.11 1.7 [0.9 – 3.1]	60.3% (38/63)	0.95 1.0 [0.5 – 1.8]	42.9% (27/63)	**0.004 2.4 [1.3 – 4.4]**	22.2% (14/63)	**<0.001 1.7 [0.8 – 3.9]**
	No	60.2% (112/186)		60.8% (113/186)		23.7% (44/186)		6.5% (12/186)	
Fever	**Yes**	46.2% (6/13)	0.24 0.5 [0.2 – 1.5]	53.8% (7/13)	0.61 0.7 [0.2 – 2.3]	46.2% (6/13)	0.2 2.3 [0.7 – 7.0]	38.5% (5/13)	**0.006 6.4 [1.9 – 21.3]**
	No	64% (151/236)		61% (144/236)		27.5% (65/236)		8.9% (21/236)	
Vomiting	**Yes**	63% (17/27)	0.99 1.0 [0.4 – 2.3]	59.3% (16/27)	0.88 0.9 [0.4 – 2.1]	37% (10/27)	0.37 1.6 [0.7 – 3.6]	14.8% (4/27)	0.50 1.6 [0.5 – 5.0]
	No	63.1% (140/222)		60.8% (135/222)		27.5% (61/222)		9.9% (22/222)	

*: Diagnosis by molecular biology (nested PCR and real-time PCR).

In the group composed of 151 *D. fragilis*-infected children, abdominal pain was the most common symptom, reported by 49.7% (75/151) of the children, followed by diarrhea, vomiting and fever in 25.2% (38/151), 10.6% (16/151), and 4.6% (7/151) of cases, respectively. Of the total of 151 *D. fragilis*-infected children, 71 were asymptomatic. Univariate logistic regression analysis showed that contact with parents having gastrointestinal symptoms (OR: 2.2, CI: 1.2-3.9 P: 0.01) was the only risk factor associated with the presence of this parasite. *D. fragilis*-infected children were 4 times more likely to be infected with *Blastocystis* sp. (OR: 3.6 CI: 2.1-6.3, P<0.001) (Table 2).

In *G. duodenalis*-infected children, abdominal pain was the most common symptom (71.8% (51/71) of children), followed by diarrhea, vomiting and fever in 38% (27/71), 14.1% (10/71), and 8.5% (6/71) of cases, respectively. Out of 71 *G. duodenalis*-infected children, 18 were asymptomatic. The logistic regression analysis found significant associations between G. *duodenalis* infection and eating raw vegetables and fruits (OR: 2.7, CI: 1.2-6.2, P :0.01), contact with parents having gastrointestinal symptoms (OR: 4.9, CI: 2.7-8.9, P <0.001), and presence of gastrointestinal symptoms (OR:4.3, CI: 2.8-8.0, P <0.001) such as abdominal pain (OR:4.7, CI:2.6-8.5, P <0.001) and diarrhea (OR:2.4, CI:1.3-4.4, P :0.004). On the other hand, HSES (OR: 0.3, CI: 0.2-0.6, P<0.001), eating out of home (OR=0.3, CI: 0.1-0.7, P: 0.003), and drinking treated water (OR: 0.3, CI: 0.1-07, P: 0.003) were protective factors against *G. duodenalis* infection (Table 2).

In *Cryptosporidium* spp.-infected children, abdominal pain was the most common symptom, reported by 57.7% (15/26), followed by diarrhea, fever and vomiting in 53.8% (14/26), 19.2% (5/26) and 15.4% (4/26) of cases, respectively. Out of 26 *Cryptosporidium* spp.-infected children, 27% (7/26) were asymptomatic. The univariate logistic regression analysis showed that children aged under 5 years had a 6 times higher risk of *Cryptosporidium* spp. infection compared with older children (OR: 6.4, CI: 1.9 – 21.3, P: 0.006). Eating outside the home (OR: 2.4, CI: 1.1-5.6, P: 0.04) and presence of gastrointestinal symptoms (OR: 3.1, CI: 1.2-7.6, P: 0.01), especially diarrhea (OR: 4.1, CI: 1.8-9.5, P <0.001) or fever (OR: 6.4, CI: 1.9-21.3, P: 0.006) were other factors significantly associated with this infection (Table 2).

Species identification and subtyping

The real-time PCR products of the 157 samples positive for *Blastocystis* sp. were sequenced on both strands. In total, with more than 99% sequence identity to the reference sequences, 138 isolates were identified as monoinfections, and 3 different STs were identified as follows:

ST3 (46.3% of isolates), ST2 (28.3%) and ST1 (25.4%). After sequence analysis, 9 samples showed mixed infections with at least two different STs that were not identified.

In addition, the PCR products of the 26 samples positive for *Cryptosporidium* spp. were successfully sequenced on both strands. Among them, 20 isolates (77%) were identified as *C. hominis*, while 6 isolates (23%) were identified as *C. parvum*, all with more than 99% sequence identity to homologous sequences. *Cryptosporidium* spp. other than *C. parvum* and *C. hominis* were not found. Sequence analysis of the gp60 gene identified the *C. hominis* isolates as belonging to two subtypes: IaA18R3 (4/20) and IbA10G2 (16/20). All of the *C. parvum* isolates were identified as the IIaA15G1R1 subtype.

Discussion

Prevalence of protozoan infections

This study demonstrates that protozoan parasitic infections are very common among a community of children living in Tripoli, independently of their socioeconomic status. This prevalence is high, considering that the study was performed in an urban area and relied on the collection of a single fecal sample per child, instead of the ideal three consecutive samples. A recent study in Malaysia among schoolchildren reported a prevalence of parasitic infections of 98%, but in a mainly rural area (Al-Delaimy et al., 2014).

The most frequent intestinal parasites detected were *Blastocystis* sp. and *D. fragilis*, followed by *G. duodenalis* and *Cryptosporidium* spp. These 4 protozoa were detected by molecular tools, which are advantageous due to their high sensitivity and specificity. DLM was performed in order to detect co-infection with additional parasites. Helminths were detected by microscopic observation with a lower prevalence, but although microscopic detection of helminths is widely used as a diagnostic method, microscopy is not very sensitive when infections are light, especially in asymptomatic persons. In addition, specific techniques for the diagnosis of certain nematodes were not used. For instance, the Scotch test was not performed for the specific detection of *Enterobius vermicularis*.

Today, *Blastocystis* sp. is considered an emerging parasite, with a worldwide distribution and prevalence in humans far exceeding that of other intestinal parasites (Alfellani et al., 2013a; Tan, 2008). In a previous study of our group, a lower prevalence of 19% was found in a population of Lebanese symptomatic and asymptomatic patients after microscopic examination of stools (El Safadi et al., 2013). In the present study, after an analysis with

molecular tools, 63% of children were found to be infected with *Blastocystis* sp. Indeed, its prevalence can reach 100% in developing countries and it has been reported between 1.5% and 20% in industrialized countries (El Safadi et al., 2014; Tan, 2008). The current prevalence of *Blastocystis* sp. among schoolchildren was high, as observed in other countries such as Senegal (100%) [7], Egypt (33%) (Rayan, Ismail, and El Gayar, 2007), Syria (28%) (Al-kafri and Harba, 2009), the USA (23%) (Amin, 2002), and Pakistan (17%) (Mehraj et al., 2008). A comparison of the prevalence of parasitic infections in different countries is shown in Table 3.

Table 3. Prevalence of parasites detected in stool samples of schoolchildren in Tripoli, and comparison with results from other studies in developing countries

Country Year	Lebanon 2004	Lebanon 2008	Pakistan 2008	India 2012	Cuba 2012	Qatar 2010	Yemen 2011	Malaysia 2014	Iran 2010	Lebanon 2015
Target population	Hospitalized patients*	Healthy adult workers*	Children*	Hospitalized patients*	Children*	Hospitalized patients*	Hospitalized patients*	Children*	Mentally retarded children*	Children (current study)
Reference	(Hamze et al., 2004)	(Hamze, Naja, and Mallat, 2008)	(Mehraj et al., 2008)	(Parameshwarappa, Chandrakanth, and Sunil, 2012)	(Canete et al., 2012)	(Abu-Madi, Behnke, and Doiphode, 2010)	(Alyousefi et al., 2011)	(Al-Delaimy et al., 2014)	(Tappeh Kh et al., 2010)	
					Prevalence (%)					
Any parasite	33.35	57.8	52.8	27.6	71.1	10.2	40.3	98.4	20.4	85.1
Blastocystis sp.	ND	ND	10.1	ND	40	4.3	ND	15.1	4	63
D. fragilis	ND	37.5	ND	ND	ND	ND	ND	ND	ND	60.6
G. duodenalis	5.1	3.2	28.9	2.4	57	1.9	17.7	28.3	6.2	28.5
E. histolytica/ dispar	ND	6.5	ND	18.1	2	0.3	17.1	12	0.4	5.6
Cryptosporidium spp.	ND	ND	ND	0	ND	ND	1	5.2	ND	10.4
E. coli	12.8	28.6	2.3	0	3	0	ND	15.5	9.7	2.4
A. lumbricoides	12.4	4.5	16.5	3.5	7	0.3	2.4	47.8	0	0.4
H. nana	0.1	0	0.9	0.3	ND	0.1	1.4	ND	0	0.4
Taenia spp.	1.1	0.3	ND	0.5	ND	ND	0	ND	0	0
E. vermicularis	0.1	ND	ND	0.1	2	ND	0.4	ND	3.4	0
T. trichiura	0.03%	ND	ND	0.2	3	ND	ND	96	ND	0

ND: Not determined

*: Detection of parasites based on microscopy

Using PCR tools, the prevalence of *D. fragilis* reached 61%. This protozoan has a worldwide distribution, with prevalence rates varying widely from 0.2% to 83% (Barratt et al., 2011). A previous study using microscopic techniques reported a prevalence of 38% of *D. fragilis* in

adult workers in the food sector, in the same geographic area of Lebanon (Hamze, Naja, and Mallat, 2008). In addition, in our study, we found a significant association between *Blastocystis* sp. and *D. fragilis* co-infection in children (P<0.001). An association between *Blastocystis* sp. and *D. fragilis* has recently been reported in children presenting gastrointestinal symptoms in the Netherlands (Maas et al., 2014) and in asymptomatic people in two poor communities in Brazil (David et al., 2015).

G. duodenalis is one of the most common causes of waterborne disease outbreaks associated with drinking water (Baldursson and Karanis, 2011; Yoder et al., 2012). The prevalence of giardiasis ranges between 0.4 to 7.5% in developed countries, and can reach 30% in developing countries (Feng and Xiao, 2011). Recent studies in asymptomatic children around the world reported giardiasis prevalence ranging from 1% in the USA (Amin, 2002), 1% in Italy (Guidetti, Ricci, and Vecchia, 2010), 1% in the United Kingdom (Davies et al., 2009), 2% in Germany (Sagebiel et al., 2009), 7% in Portugal (Julio et al., 2012), 7% in Pakistan (Mehraj et al., 2008), 15% in Syria (Al-kafri and Harba, 2009), 18% in Yemen (Alyousefi et al., 2011), 32% in Russia (Kramar, Reznikov, and Kramar, 2003) and 57% in Cuba (Canete et al., 2012). An overall prevalence of 29% was found in our study. This prevalence is considerably higher than that in other Middle Eastern countries with similar levels of welfare (Table 3) or in European countries (i.e. Italy, Germany, the UK, Portugal) (Ryan and Caccio, 2013). In addition, the current prevalence of giardiasis in Lebanon is six times higher than that observed in 2004 (5%) (Table 2) (Hamze et al., 2004) and the higher sensitivity of molecular tools for the detection of this parasite could explain this difference.

Regarding *Cryptosporidium* spp., this apicomplexan protozoan is one of the most common intestinal parasitic pathogens in the world (Chalmers and Katzer, 2013). This parasite is a serious cause of diarrheal disease and a major concern for the production of safe drinking water (Baldursson and Karanis, 2011). An epidemiological study including 22,500 infants and children showed that *Cryptosporidium* spp. were one of the four pathogens responsible for severe diarrhea (Kotloff et al., 2013), and were considered the second most significant cause of diarrhea and death in children after rotavirus (Striepen, 2013). Cryptosporidiosis rates are higher in children and immunocompromised patients than in the healthy adult population (ANOFEL, 2010). However, cryptosporidiosis prevalence varies in different countries: between 1% and 5% in children with diarrhea in developed countries, reaching 49% in developing countries (ANOFEL, 2010; Cardona et al., 2011; Helmy et al., 2013). The prevalence that we found in children in Lebanon (10%) was in the same range as that

observed in Yemen (10%) (Alyousefi et al., 2013), but lower than that found in others Middle Eastern countries such as Jordan (19%) (Hijjawi et al., 2010) and Egypt (49%) (Helmy et al., 2013).

Entamoeba histolytica/dispar are found in all parts of the world, but most frequently in geographic areas with low socioeconomic status and poor environmental sanitation (Noor Azian, Lokman Hakim, and Maslawaty, 2006; Qvarnstrom et al., 2005). It has been estimated that infection with *E. histolytica* results in 34 million to 50 million symptomatic cases of amebiasis worldwide each year, causing 40 to 100 thousand deaths annually (Tanyuksel and Petri, 2003). *E. dispar* is not pathogenic compared to *E. histolytica*, but it is not possible to differentiate between these two parasites using only light microscopy if ingested red blood cells are not observed inside the *E. histolytica* trophozoites (Costa et al., 2006; Tan et al., 2010). Even though we did not use PCR to detect this parasite, our results showed that infection with *E. histolytica/dispar* is more prevalent in Lebanon nowadays (6%) compared to previous results (2%) obtained in 2004 (Saab et al., 2004) (Table 3). It is also more prevalent than in other Middle Eastern countries, such as Syria (0.01%) (Al-kafri and Harba, 2009), Qatar (0.3%) (Abu-Madi, Behnke, and Doiphode, 2010) and Iran (0.4 - 2%) (Pestehchian et al., 2011; Tappeh Kh et al., 2010), and in other developed (Amin, 2002) and developing countries (Escobedo, Canete, and Nunez, 2008). Nevertheless, the parasite is less common than in other developing countries like Pakistan (14%) (Mehraj et al., 2008), Yemen (17%) (Alyousefi et al., 2011), and India (18%) (Parameshwarappa, Chandrakanth, and Sunil, 2012).

Concerning the high prevalence of co-infections of pathogenic and nonpathogenic parasites, our results are comparable to those of other studies (Hurlimann et al., 2014; Ouattara et al., 2010). The observed polyparasitism could be explained by shared risk factors for parasite infection, such as poor sanitation and hygiene behavior and by the fact that the transmission route of these parasites is mainly through the fecal-oral pathway (Hurlimann et al., 2014).

Clinical manifestations and associated risk factors

In total, 125 children out of 249 had symptoms at the time of the survey. In relation to the main clinical features of infections, it was found, as expected, that diarrhea was significantly common among *G. duodenalis* and *Cryptosporidium* spp.-infected children, but no significant association with this symptom was observed regarding *Blastocystis* sp. or *D. fragilis* infections. The interactions and confounding effects that are not evident in a simple comparison of the two groups could also explain the absence of significant associations.

Nevertheless, a positive association regarding *Blastocystis* sp. and abdominal pain suggests a pathogenic role for this parasite of controversial clinical significance (Mehlhorn, Tan, and Yoshikawa, 2012). Even if children harboring *D. fragilis* presented more gastrointestinal symptoms, no significant association was found between this parasite and gastrointestinal disorders in children. Recent studies described that *D. fragilis* has struggled to gain recognition as a pathogen, despite the evidence supporting its pathogenic nature (Stark et al., 2010). Interestingly, the 124 other children were asymptomatic for protozoan infection and may represent carriers responsible for transmission.

Concerning the risk factors for protozoan infection, our data analysis found that protozoan parasites could infect both genders in all age groups. However, an age of less than 5 years was significantly associated only with *Cryptosporidium* spp. infection. The reason for this high prevalence is likely due to the immature immunity of young children exposed to this opportunistic parasite (Fournet et al., 2013). As reported by other authors, no association was found between either gender or age and prevalence of *G. duodenalis* infection (Julio et al., 2012). It is not yet fully understood why age plays a role in the frequency of *Cryptosporidium* infection, but is not associated with the frequency of giardiasis (Nundy et al., 2011).
Intestinal parasites are usually considered poverty-related diseases (Osei-Atweneboana et al., 2012). However, no significant association was identified between socioeconomic status and the overall rate of parasitic infections in our study population. Nevertheless, the prevalence of *G. duodenalis* was significantly higher in LSES infected children. Interestingly, in a previous study conducted in Peru, *Giardia* sp. and microsporidia were the predominant intestinal parasites among the poorest population, and infections with *Cryptosporidium* spp. were independent on wealth (Nundy et al., 2011). Furthermore, in our study, only LSES children were infected with helminths (*Ascaris lumbricoides* and *Hymenolepis nana*).

We also showed that children who drank untreated water had a 3 times higher risk of infection with *G. duodenalis* than those who drank treated water (P: 0.003). Two meta-analyses including 84 studies in 28 countries concluded that the quantity of water available to the population in developing countries has more impact on endemic diarrhea cases than water purity itself (Esrey et al., 1989; Esrey, Feachem, and Hughes, 1985). For the study population in Lebanon, the accessibility of the water supply was not a problem. However, a majority of households did not have a proper sanitary system, favoring fecal contamination via ground seepage, as previously described (Schmidt, Al-Nozaily, and Al-Ghorbany, 2008).

The findings of this study showed that children who had contact with family members presenting gastrointestinal symptoms had a higher risk of infection with these parasites, confirming the direct human-to-human transmission of these protozoa. Thus, the screening and treatment of family members of infected children should be considered for the prevention and control of these infections. Additionally, indirect transmission through contaminated food (raw vegetables and fruits) was found to be a risk factor for giardiasis. In fact, this association is possible due to the fact that fresh vegetables and fruits may be eaten without washing them or with contaminated hands, and it is well known that contaminated hands can play a major role in fecal-oral transmitted diseases [44]. A significant negative association was observed between infection either with *G. duodenalis* or *Blastocystis* sp. and meals eaten outside of the household. This could be a cofounding factor, due to the fact that only one child in the LSES group reported eating outside the home. On the other hand, meals outside the home were significantly associated with *Cryptosporidium* spp. infection.

Subtyping of *Blastocystis* sp. and *Cryptosporidium* spp. isolates

The subtyping of *Blastocystis* sp. and *Cryptosporidium* spp. isolates allows an elucidation of the transmission of these 2 parasites. The majority of *Blastocystis* sp. positive samples included in this study represented monoinfections (88%) by one ST. Among these positive isolates, three STs were detected as follows: ST3 was the most abundant, followed by ST2 and ST1 (35/138). Our previous study in the Lebanese population also identified the same three STs, with a predominance of ST3 and ST2 (El Safadi et al., 2013). Interestingly, the majority of human *Blastocystis* sp. infections around the world are attributed to ST3 isolates, followed by ST1 and ST2, which is consistent with spread directly from person to person (Stensvold, 2013). Interestingly, ST4 was not found in our study. Overall, this ST is common in Europe, but much less frequent in Lebanon as well as in Middle Eastern, African, American and Asian countries (Stensvold, 2013). In our cohort of schoolchildren, molecular characterization of *Cryptosporidium* spp. isolates allowed the identification of *C. parvum* and *C. hominis*, with a predominance of the latter. It is well known that human cryptosporidiosis is mainly caused by these two species, with *C. parvum* considered a zoonotic species while *C. hominis* has been mainly associated with anthroponotic transmission (Chalmers and Katzer, 2013). Consistently, a potential secondary transmission of infection among family members was significantly associated with this infection.

These results are consistent with our recent study describing the predominance of *C. hominis* in Lebanese hospitalized patients (Osman et al., 2015). However, we found different subtypes

than those reported in the previous study from our group (Osman et al., 2015). Two subtypes belonging to the subtype families Ia and Ib, IaA18R3 and IbA10G2, were identified. The subtype IdA19, which has been described as the predominant subtype in Lebanese hospitalized patients (Osman et al., 2015), was not found in schoolchildren. The subtype family IbA10G2 has been commonly reported around the world, and is the predominant cause of waterborne outbreaks due to *C. hominis* (Chalmers, 2012). However, IaA18R3 is a rare subtype recently reported in India and Spain (Fuentes et al., 2014; Sharma et al., 2013). All subtyped *C. parvum* isolates were identified as the IIaA15G1R1 subtype. This zoonotic subtype has been reported in both humans and animals in many geographic areas of the world (Ryan, Fayer, and Xiao, 2014). Moreover, the *C. parvum* IIa subtype family has a high genetic diversity, and is responsible for the majority of cryptosporidiosis outbreaks due to *C. parvum* (Chalmers, 2012). However, the IIc and IId subtype families, which are reported mostly in developing countries, have not been described in Lebanon (Adamu et al., 2014; Helmy et al., 2013; Hijjawi et al., 2010; Nazemalhosseini-Mojarad et al., 2011).

Conclusions

To our knowledge, this is the first study reporting epidemiological data on intestinal protozoan infections among schoolchildren in Lebanon, independently of socioeconomic status. Our results showed a high prevalence of protozoan parasites among this population, *Blastocystis* sp. being the most predominant protozoa. In addition, although 50% of children reported symptoms, many of them were asymptomatic, and these children could serve as unidentified carriers. Contact with family members with gastrointestinal disorders was found to be the main risk factor associated with the presence of protozoan infections. The role of person-to-person contact in the specific transmission of *Blastocystis* sp. and *Cryptosporidium* spp. isolates was consistent with the results of subtyping. The findings of this study provide useful information for the design of prevention strategies, and interventions in target communities at risk.

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2. Axe 2 : Etude de la prévalence de *Cryptosporidium* spp. dans les échantillons animaux et l'évaluation du pouvoir zoonotique du parasite.

1. Article 3 :

Titre: « Cryptosporidiosis in humans and cattle in a rural area of Northern Lebanon ».

Préambule : Cet article est en cours de préparation.

Résumé :

Les infections parasitaires constituent un problème majeur dans le monde. La prévalence des infections parasitaires dans les populations humaines est beaucoup plus importante dans les pays en développement comparativement aux pays développés car on se heurte dans ces régions à de nombreux facteurs défavorables, dont la pauvreté, la consommation d'aliments contaminés et la pollution de l'eau. Outre son impact en santé humaine la cryptosporidiose est la cause majeur de diarrhée chez les jeunes animaux d'élevage ce qui conduit à des pertes économiques non négligeables. Le but de cette étude a été de déterminer une prévalence précise de *Cryptosporidium* spp. en utilisant des techniques moléculaires tout en clarifiant la circulation du parasite dans les populations humaines et bovines du district d'Akkar, région géographique rurale du Nord-Liban.

Cent selles de patients, d'âge compris entre 4 mois et 88 ans, atteints ou non de pathologies intestinales ont été collectées dans les hôpitaux de cette région (Hôpital Rahal d'Akkar; et Hôpital Al-Youssef d'Akkar). Du fait du fort potentiel zoonotique de certaines espèces et sous-types du parasite, 152 selles de bovins vivants dans cette région ont aussi été collectées. L'ADN total a été extrait des selles humaines et animales à l'aide du Mini kit QIAamp DNA stool (Qiagen®). Tous les ADN extraits ont été stockés à -20°C avant d'être génotypés en France. La détection de *Cryptosporidium* dans tous les échantillons a été réalisée par PCR nichée en ciblant l'ADNr 18S. Tous les produits de PCR ont été séquencés. Afin d'avoir plus d'information sur la transmission de l'infection, le polymorphisme génétique des isolats de *C. parvum* et *C. hominis* a été étudié à l'aide du marqueur moléculaire gp60. Les résultats obtenus sont les suivants :

1. L'analyse moléculaire a permis de mettre en évidence d'une part la présence de *Cryptosporidium* dans 5 échantillons humains ce qui représente une prévalence de 5% du parasite chez l'homme dans le district d'Akkar.

2. Il est à noter que les cinq échantillons positifs correspondent à des patients symptomatiques.

3. D'autre part, *Cryptosporidium* a été identifié dans 12 échantillons de bovins (8%).

4. Chez l'homme deux espèces ont été identifiées, *C. hominis* (80%) et *C. parvum* (20%). Alors que trois espèces ont été identifiées chez les bovins : *C. andersoni* (50%), *C. bovis* (33%) et *C. parvum* (17%).

5. L'analyse du locus gp60 des isolats de *C. hominis* et *C. parvum* a montré la présence des sous-types IdA19 de *C. hominis* et IIaA15G1R1 de *C. parvum* chez l'homme et des deux sous-types IIaA15G1R1 et IIaA15G2R1 de *C. parvum* chez les bovins. Ainsi, le sous-type IIaA15G1R1 de *C. parvum* est retrouvé aussi bien chez l'homme que chez les bovins.

Cette étude a eu certaines limites, telles que :

1. L'échantillonnage des selles d'origine humaine n'a pu être réalisé qu'au niveau des hôpitaux régionaux et pas au niveau de la population générale.

2. Des raisons logistiques ont limité l'échantillonnage des selles provenant de jeunes animaux.

Cependant**,** ces résultats ont permis de rapporter les premières données d'épidémiologie moléculaire sur la cryptosporidiose chez les bovins au Liban. Ces données chez l'homme et les bovins nous laissent présager une prédominance du mode de transmission anthroponotique sans exclure pour autant une transmission zoonotique. Les bovins seraient donc des réservoirs animaux de contaminations potentielles pour l'homme.

Ma contribution dans cette étude a été la suivante:

- Conception de l'étude
- Réalisation des expériences
- Analyse des données
- Rédaction de l'article

Cryptosporidiosis in humans and cattle in a rural area of Northern Lebanon

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Abstract :

Cryptosporidium apicomplexan protozoa are ubiquitous intracellular agents affecting humans and animals worldwide. A previous study of our group reported a high prevalence of these parasites in Lebanese symptomatic hospitalized patients in North Lebanon. However, no data are currently available about cryptosporidiosis among animals in this country. To improve our understanding of the epidemiology of cryptosporidiosis, the main aim of this study was to determine the prevalence and the genetic diversity of *Cryptosporidium* in both human and cattle populations in the rural district of Akkar in North Lebanon. Fecal specimens were collected from 100 randomly selected hospitalized patients in different medical departments in two hospitals (Al-Youssef Hospital and Rahal Hospital), and from 153 Holstein cattle from 33 farms or barns, in the Akkar district. The overall prevalence of *Cryptosporidium* spp. infection obtained by molecular analysis was 5% and 8% in humans and cattle, respectively. Among *Cryptosporidium* isolates in humans, 4 (80%) were identified as *C. hominis*, while the remaining 1 (20%) was identified as *C. parvum*. In cattle *C. andersoni* was predominant (50%) followed by *C. bovis* (33%) and *C. parvum* (17%). After analysis of the gp60 locus, *C. hominis* IdA19, a rare subtype, was found to be predominant. Two *C. parvum* subtypes were found in humans and cattle: IIaA15G1R1 and IIaA15G2R1. These epidemiological results predict that both anthroponotic and zoonotic transmissions occur in the Akkar district.

Introduction:

Cryptosporidium are worldwide intestinal opportunistic protozoan parasites that infect humans as well as a broad spectrum of domestic and wild hosts including ruminants, carnivores, and primates (Ryan, Fayer, and Xiao, 2014). *Cryptosporidium* parasites have low infective doses as showed in human volunteers (Chappell et al., 2006; Okhuysen et al., 1999) and animal models (Benamrouz et al., 2014), and oocysts are very resistant to environmental and water treatment (Ryan, Fayer, and Xiao, 2014). *Cryptosporidium* oocysts have the potential to be transmitted by fecal-oral route and can be also spread by close proximity to infected humans and animals (Ryan, Fayer, and Xiao, 2014). The importance of *Cryptosporidium* in human and animal health has long been underestimated. However, *Cryptosporidium* is increasingly recognized as one of the major causes of moderate to severe diarrhea, especially in developing countries (Ryan, Fayer, and Xiao, 2014). An epidemiological study of more than 22000 infants and children in Africa and Asia found that this parasite was the second pathogen responsible for severe diarrhea (Kotloff et al., 2013) and was considered the second greatest cause of death in children after rotavirus (Kotloff et al., 2013; Striepen, 2013).

In addition, *Cryptosporidium* infections are described as important and established cause of cattle morbidity, weight loss, delayed growth and sometimes high mortality among farm animals (de Graaf et al., 1999). The species *C. parvum* and *C. hominis,* are major causes of human diarrhea worldwide (Fayer, 2010). Whereas *C. hominis* is predominantly found in humans, cattle, especially newborn calves, are important reservoirs for *C. parvum*, and contact with cattle has been implicated as a risk factor for human cryptosporidiosis in different countries (Ryan, Fayer, and Xiao, 2014) via direct transmission or indirectly, through the deposition of fecal material into water sources or agricultural land (Ehsan et al., 2015). Additionally, cattle can be infected with other *Cryptosporidium* species such as *C. andersoni*, *C. bovis* and *C. ryanae* (Ryan, Fayer, and Xiao, 2014), and these species with the exception of *C. ryanae* have been reported among human cases (Chalmers and Katzer, 2013).

In a recent study from our group, *Cryptosporidium* spp. was identified as a prevalent parasite in humans in Lebanon, and among infected patients, *C. hominis* and *C. parvum* were identified as the species responsible for the infection (Osman et al., 2015). However, to our knowledge no studies are available on the epidemiology of *Cryptosporidium* in animals, and in particular in cattle in this country.

Therefore, in order to better understand the transmission of cryptosporidiosis in a rural region of Lebanon, the aim of this study was to determine the prevalence of *Cryptosporidium* and the genetic diversity of isolates infecting the inhabitants and animals in the rural district of Akkar, (North of Lebanon). In this region, one of the poorest of Lebanon (FAO, 2014) a prevalence of 11% of *Cryptosporidium* in diarrheic hospitalized patients was previously reported (Osman et al., 2015).

Materials and Methods:

Ethics Statement

Permission for this study was obtained from the Minister of Public Health (reference number: 4-39716). Protocol of this project was in agreement with the Lebanese reglamentation. Written informed consents were obtained from the parents or the legal guardians of the children or directly from adult patients participating in the study after a clear explanation of the research objectives. Verbal consent of animal owners was obtained prior to the collection of fecal samples. The present study was in accordance with the Code of Ethics of the World Medical association (Declaration of Helsinki), and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

Sample collection

Fecal specimens were collected in two hospitals (Al-Youssef Hospital and Rahal Hospital) from 100 hospitalized patients randomly selected in different medical departments among patients presenting or not gastro-intestinal symptoms (62 males and 38 females, ranging in age from 4 months to 88 years with a mean age of 11 years), and in 33 farms or barns located in 20 villages of the Akkar district, from 153 Holstein cattle (majority of animals were adults) during the period of November-December 2013. One fecal sample per patient and animal was collected in a sterile container. Animal specimens were collected directly from the rectum of each animal with the assistance of a veterinarian. Samples were immediately transported to the laboratory after collection.

DNA extraction, species identification and subtyping

DNA was extracted from approximately 250 µg of fecal samples using the QIAmp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommended procedures. The DNA was eluted in 100 µl of elution buffer (Qiagen) and stored at −20 °C until use. The 18S rRNA nested PCR was performed with primers, as

previously described by Xiao *et al*. (Xiao et al., 1999). To test the potential presence of PCR inhibitors in fecal specimens, 0.5 µl of pure DNA of *C. parvum* was added to negative DNA samples and processed by PCR. To identify *Cryptosporidium* spp. molecularly, positive PCR products were purified and sequenced directly by the company Genoscreen (Pasteur Institute, Lille) on both strands using the forward and reverse primers used for the nested (secondary) PCR. The sequences obtained were aligned using the BioEdit v7.0.1 package (http://www.mbio.ncsu.edu:BioEdit/bioedit.html), then compared with sequences of *Cryptosporidium* published on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) using the basic local alignment search tool (BLAST) program. Specimens genotyped as *C. parvum* or *C. hominis* were further subtyped using a second nested PCR, which amplifies a fragment of the 60 kDa glycoprotein (gp60) gene, widely used in *Cryptosporidium* subtyping because of its sequence heterogeneity and relevance to parasite biology, as described previously (Alves et al., 2003). The amplified DNA fragments were purified, sequenced, and analyzed by alignment of gp60 sequences with reference sequences retrieved from GenBank using the program ClustalX (http://www.clustal.org/). *C. parvum* and *C. hominis* gp60 subtypes were named by counting the number of trinucleotide repeats of TCA (A), TCG (G), and TCT (T) and the ACATCA repeat (R) after the trinucleotide repeats (Alyousefi et al., 2013; Sulaiman et al., 2005).

Results:

***Cryptosporidium* prevalence**

A total of 253 stool samples were examined by molecular analysis, 100 human and 153 animal samples. Out of human feces, 5 (5%) samples only from children of < 2 years old were positive for *Cryptosporidium* spp.. All these *Cryptosporidium* infected children presented gastrointestinal disorders such as abdominal pain, diarrhea and vomiting. However, no significant association was found between *Cryptosporidium* positivity and age, sex or symptoms. The overall prevalence of *Cryptosporidium* infected animals was 7.8% (12/153).

***Cryptosporidium* species identification**

For species identification, the positive nested PCR products were sequenced. Sequence analysis from *Cryptosporidium* positive patients revealed the presence of *C. hominis* (4/5) and *C. parvum* (1/5). The distribution of *Cryptosporidium* species identified in animals was as follows: the species *C. andersoni*, *C. bovis* and *C. parvum* were found in 50%, 33.3% and 16.7% of cases, respectively.

***Cryptosporidium* subtyping by gp60 analysis**

The partial sequence of the gp60 gene was subsequently obtained for 4 *C. hominis* and 3 *C. parvum* (one from human and two from animal isolates). All *C. hominis* isolates were identified as IdA19 subtype. The human *C. parvum* isolate was subtyped as IIaA15G1R1 and the two cattle subtypes of *C. parvum* belonged to the subtype family IIa and were identified as IIaA15G1R1 and IIaA15G2R1.

Discussion:

In the present study, the prevalence of *Cryptosporidium* species in Akkar patients after molecular analysis was 5%, lower to that found before in the same region in a previous study from our group (Osman et al., 2015). This low prevalence reported in general population confirm that symptomatic patients have an increased risk of cryptosporidiosis (Hunter and Nichols, 2002). All cases of cryptosporidiosis were detected among children less than 2 years old. This prevalence of cryptosporidiosis in humans is lower than that reported in several neighboring developing countries (Abu-Alrub et al., 2008; Alyousefi et al., 2013; Helmy et al., 2013; Hijjawi et al., 2010; Shalaby et al., 2014; Usluca and Aksoy, 2011). However, this frequency is higher than that reported in developed countries such as France or Spain (ANOFEL, 2010; Cardona et al., 2011). Unfortunately, one limitation of our study was the impossibility of including animal handlers in the sampling. The analysis of this population could contribute to detect a higher prevalence of *Cryptosporidium* in this region but due to cultural reasons is difficult to obtain fecal samples from healthy persons in Lebanon.

In the same context, the prevalence of *Cryptosporidium* in cattle was 8%, which is lower than prevalence reported in epidemiological studies in neighboring countries (Helmy et al., 2013; Keshavarz et al., 2009). However, one of the limitations of this study is that most of the examined animals were adults due to logistic reasons that restricted the sampling of young animals. Indeed, several studies about cryptosporidiosis in cattle have reported a host age-related susceptibility being the infection more frequent in pre-weaned calves (< 8 weeks) (Follet et al., 2011; Santin et al., 2004; Wang et al., 2011). These results are in consistent with the fact that *C. andersoni* was the predominant species. Actually, it has been reported that *C. andersoni* seems to be the predominant species in adult cattle (Wang et al., 2011).

After analysis of a fragment of the gp60 gene, our data showed that subtyped *C. hominis* isolates from humans belonged to the Id subtype family and were identified as IdA19 subtype. Even if the subtype family Id has been commonly reported around the world (Xiao, 2010), the

subtype IdA19, previously described as predominant in Lebanese hospitalized patients (Osman et al., 2015), is less common. It has been detected to our knowledge only in Canada (Trotz-Williams et al., 2006) and China (Feng et al., 2012).

The role of cattle in the zoonotic transmission of *C. parvum* in the Akkar region was further supported by gp60 subtyping data. Three isolates of *C. parvum* identified in human (1/3) and cattle (2/3) belonged to the IIa subtype family. The *C. parvum* IIa subtype family has a high genetic diversity and is responsible of the majority of cryptosporidiosis outbreaks due to *C. parvum* (Xiao, 2010). On the other hand, the *C. parvum* IId subtype family presenting a high frequency in several countries in Middle East, was not detected in Lebanon (Helmy et al., 2013; Hijjawi et al., 2010; Iqbal, Khalid, and Hira, 2011; Nazemalhosseini-Mojarad et al., 2011). Neither the IIc anthroponotic subtype family of *C. parvum*, which has an important presence in other developing countries like India, Jordan, Kuwait, South Africa, Uganda, Jamaica, and Peru (Hijjawi et al., 2010; Iqbal, Khalid, and Hira, 2011; Xiao, 2010). Particularly, 2 subtypes were found: IIaA15G1R1 and IIaA15G2R1, and the first one was common between human and cattle. Nevertheless, both subtypes have been reported in human and animals in many geographic areas of the world (Xiao, 2010), as well as in our study of a cohort of a symptomatic population in Lebanon (Osman et al., 2015).

As a risk factor for human cryptosporidiosis, direct contact with cattle has been implicated in other countries (Xiao and Feng, 2008). However, in the present study, taking into account the young age of the infected child, contact with livestock seems improbable as it was reported in Tunisia (Rahmouni et al., 2014). Then, other probable risk factors for *C. parvum* transmission in this area such as drinking raw (unpasteurized) milk or drinking contaminated water should be explored (Rahmouni et al., 2014).

In conclusion, despite the low number of samples, these results represent the first molecular characterization of *Cryptosporidium* in animals in Lebanon. Epidemiological and molecular evidence predict that anthroponotic and zoonotic transmissions occur in the Akkar district. More research involving more human, animals and environmental samples are recommended in order to better identify risk factors and characterize the transmission modalities and routes of the infection.

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Competing Interests

The authors have declared that no competing interests exist.

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2. Article 4 :

Titre: « Prevalence and genetic diversity of the intestinal parasites *Blastocystis* sp. and *Cryptosporidium* spp. in household dogs in France and evaluation of zoonotic transmission risk».

Préambule : Cette étude a fait l'objet d'un article soumis au journal « Veterinary Parasitology ». Il est actuellement en révision.

Résumé :

Plusieurs parasites, y compris les protozoaires *Blastocystis* sp. et *Cryptosporidium* spp. peuvent être responsables d'infections gastro-intestinales chez les chiens domestiques avec un risque potentiel de transmission de la maladie à leurs propriétaires. Même si la France est l'un des plus grands pays d'Europe en termes de population canine, peu de données sont disponibles sur l'épidémiologie moléculaire de ces deux parasites. Le but de cette étude était de déterminer la prévalence des parasites intestinaux chez les chiens domestiques en France, et d'évaluer le risque zoonotique de *Blastocystis* sp. et *Cryptosporidium* spp. par le génotypage des isolats correspondants.

Cent seize échantillons de selles ont été prélevés à l'école vétérinaire VetAgro Sup de Lyon en France, à partir des chiens domestiques sans distinction de race, d'âge ou de sexe, vivant dans la région, et présentant ou non des diarrhées. Divers protozoaires et helminthes intestinaux ont été identifiés par microscopie optique. Le dépistage de *Blastocystis* sp. et *Cryptosporidium* spp. a ensuite été effectué par PCR ciblant le gène codant l'ARNr 18S, suivie d'un séquençage direct des produits et de l'analyse des séquences obtenues pour le génotypage des isolats. Voici les détails des résultats les plus marquants :

1. Le taux de chiens infectés par au moins un seul parasite gastro-intestinal était de 42,2% (49/116).

2. L'examen microscopique direct des selles, a permis de montrer que les parasites les plus fréquemment trouvés étaient les protozoaires *Giardia* spp. (25%) et *Cystoisospora* spp. (19,8%).

3. Les méthodes moléculaires ont permis de révéler que quatre chiens (3,4%) étaient infectés par *Blastocystis* sp.

4. Le sous-type ST2, sous-type communément identifié dans divers groupes d'animaux ainsi que le sous type ST10 fréquemment retrouvé chez les bovins ont été identifiés.

5. Trois chiens (2,6%) se sont avérés positifs à *Cryptosporidium* et l'espèce *C. canis* espèce infectant épisodiquement les humains a été identifiée comme responsable des infections,

Cette étude a permis de rapporter les premières données d'épidémiologie moléculaire sur les parasites zoonotiques *Blastocystis* sp. et *Cryptosporidium* spp. chez les chiens domestiques en France. La faible prévalence de ces deux parasites, combiné à l'identification de *C. canis* et des sous-types ST2 et ST10 de *Blastocystis* sp. dans la population canine, suggère fortement que les chiens jouent un rôle négligeable en tant que réservoirs zoonotiques pour ces deux parasites et ne semblent pas être des hôtes naturels de *Blastocystis* sp. Aucune association avec la présence des symptômes gastro-intestinaux n'a pu être clairement établie à partir de nos données.

Ma contribution dans cette étude a été la suivante :

- Conception de l'étude
- Réalisation des expériences
- Analyse des données
- Rédaction de l'article

Prevalence and genetic diversity of the intestinal parasites *Blastocystis* sp. and *Cryptosporidium* spp. in household dogs in France and evaluation of zoonotic transmission risk

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Abstract

Background: Several parasites including the protozoa *Blastocystis* sp. and *Cryptosporidium* spp., may be causative agents of gastrointestinal symptoms in household dogs, and there may be a potential risk of transmission to owners. While France is one of the largest European countries in terms of its canine population, little data is available about the molecular epidemiology of these two parasites. The purpose of this study was to determine the prevalence of intestinal parasites in household dogs in France, and to evaluate the zoonotic risk of *Blastocystis* sp. and *Cryptosporidium* spp. by genotyping the corresponding isolates.

Methods: One hundred and sixteen faecal samples were collected from household dogs regardless of race, age or gender, living in the Lyons area, France, presenting or not presenting diarrhea. Various intestinal protozoa and helminthes were identified by light microscopy. Screening for *Blastocystis* sp. and *Cryptosporidium* spp. was subsequently performed by PCR targeting the small subunit (SSU) rDNA coding region, followed by direct sequencing of the PCR products and analysis of the sequences obtained for genotyping.

Results: The overall prevalence of dogs infected with at least one gastrointestinal parasite was 42.2% (49/116). After light microscopy examination of stools, the most common parasites found were the protozoa *Giardia* spp. (25.0%) and *Cystoisospora* spp. (19.8%). Using molecular methods, four dogs (3.4%) were shown to be infected by *Blastocystis* sp. and carried either ST2, commonly identified in various animal groups, or ST10, frequently found in bovines. Three dogs (2.6%) were positive for *C. canis*, infecting humans episodically. An association between either *Blastocystis* sp. or *C. canis* infection and gastrointestinal symptoms could not be clearly established from our data.

Conclusion: This study reports the first molecular epidemiological data regarding the zoonotic parasites *Blastocystis* sp. and *Cryptosporidium* spp. in household dogs in France. The low prevalence of both parasites, combined with the identification of *C. canis* and *Blastocystis* sp. ST2 and ST10 in the canine population, strongly suggests that dogs play a negligible role as zoonotic reservoirs for both parasites and do not seem to be natural hosts of *Blastocystis* sp.

Keywords

France, Dogs, Intestinal parasites, Prevalence, *Blastocystis* sp., *Cryptosporidium* spp., Zoonosis, Molecular epidemiology

Background

Domestic dogs are considered to be the largest group of domesticated mammals, with an estimated population of 500 million worldwide. France is the fifth largest European country in terms of its canine population, with around 7.5 million dogs and more than 20% of households owning at least one dog (FACCO, TNS, and SOFRES, 2012). Companion animals and their close relationship with humans offer several well-known benefits, but they also bring potential problems including zoonotic parasites. Consequently, these pets may represent a potential source of infection for their owners, who can be exposed through consumption of food or water contaminated with animal faeces or by direct faecal-oral transmission [2]. Several zoonotic parasites have been identified in various canine populations worldwide, including the protozoa *Giardia*, *Cryptosporidium*, *Sarcocystis*, and *Cystoisospora* (syn. *Isospora*), and the helminthes *Toxocara*, *Toxascaris*, *Ancylostoma*, *Trichuris*, *Dipylidium*, and *Spirometra* [3-16]. A majority of these parasites are described as enteropathogens and causative agents of diarrhea, especially in juvenile animals, but also in humans.

In particular, *Cryptosporidium* spp. is an apicomplexan protist known to cause self-limiting diarrhea in immunocompetent adults, or life-threatening diarrhea in immunosuppressed individuals, such as acquired immune deficiency syndrome (AIDS) patients [17]. More strikingly, this parasite is also the second biggest cause of diarrheal disease and death in children [18]. Within the genus *Cryptosporidium*, 26 morphologically similar species have been currently reported as valid, presenting varying degrees of disease severity, host specificity, and zoonotic potential [17,19]. In dogs, the prevalence of *Cryptosporidium* spp. worldwide ranges from 0 to 17% [20], with a strong predominance of *C. canis*, followed by *C. parvum* and *C. muris* [17,21,22]. Since these three *Cryptosporidium* species have also been isolated in humans with varying prevalence, infection in dogs may be a public health concern due to the potential transmission of the parasites to humans [17,20,23,24].

This already extensive list of potential zoonotic parasites infecting dogs must also be supplemented by the protozoan *Blastocystis* sp. that inhabits the gastrointestinal tract of humans and many groups of animals [25]. Indeed, this enteric parasite is currently the most common unicellular eukaryote reported in human faecal samples, since its prevalence may greatly exceed 40% in under-developed and developing countries [26-28]. *Blastocystis* sp. is

associated with a variety of non-specific gastrointestinal disorders [25,29], including diarrhea, and is probably linked to irritable bowel syndrome [30]. At the molecular level, extensive genetic diversity among *Blastocystis* sp. isolates infecting mammalian hosts has been described, leading to the identification of 17 subtypes (STs), 9 of which are found in humans with varying prevalence [31,32]. Several studies strongly support the zoonotic potential of the parasite by comparing the STs found in various animal groups including farm and zoo animals, with those identified in humans living in close contact or working with these animals [33-40]. Additionally, some recent surveys have also raised the possibility that domestic dogs could be natural hosts of *Blastocystis* sp. and potential sources of zoonotic transmission [27,33,41-44]. However, in view of the low prevalence of the parasite and the large diversity of STs observed by some authors in different canine populations worldwide, it has also been suggested that dogs do not represent an important zoonotic risk with regard to the transmission of *Blastocystis* sp. [45,46].

Despite the potential public health impact of zoonotic parasites and more particularly *Cryptosporidium* spp. and *Blastocystis* sp., very few studies have been conducted to date on the prevalence of these two enteropathogens in household dogs in France. To our knowledge, only one recent survey based on light microscopy identification studied the prevalence of *Cryptosporidium* spp. solely in puppies [11], and no data is available regarding the prevalence of *Blastocystis* sp. and the distribution of STs in dogs. Therefore, the aims of this study were: i) to determine the prevalence of *Cryptosporidium* spp. and *Blastocystis* sp. infections in domestic dogs in France; ii) to explore the genetic diversity of the corresponding parasite isolates and their potential association with clinical manifestations in these companion animals; and iii) to evaluate the risk of transmission of both parasites to humans through molecular identification of the corresponding isolates.

Methods

Study population

Between October 2012 and July 2013, a total of 116 fresh canine faecal samples were collected at the School of Veterinary Medicine Vet-Agro-Sup of Lyon. 76 of these samples were collected from household dogs owned by university students and the remaining 40 samples from animals monitored in consultation by veterinarians of the school. The study was

conducted on dogs regardless of race, age or gender. In the case of university students, the owners were required to complete a questionnaire and provide a faecal sample of their pet using a sample bag and identification tag. The collection of stools and filling out of the questionnaire was carried out directly by the veterinarian during the consultations of the remaining dogs. The questionnaire was designed to collect basic information about each participating dog concerning its age, gender, breed, and presence of diarrhea as well as its previous history of anthelminthic and antiprotozoal treatments within 6 months prior to sampling (see additional file). The participation of dogs was on the voluntary basis of their owners, who provided consent for the use of samples, and only stools collected after spontaneous defecation of the animals were analyzed. No experiments involving dogs were performed. Consequently, this study did not require full Animal Ethics Committee approval in accordance with French law.

Microscopy and DNA extraction

Faeces were collected by the owners or veterinarians on the ground, immediately after natural defecation. The samples were then taken to the parasitology laboratory of the school and analyzed within 6 hours after collection. Parasitic stool analysis was performed by a standard semi-quantitative flotation technique using saturated zinc sulfate solution (density: 1.36 mg/l), allowing the identification of eggs, cysts, and oocysts according to their morphological characteristics under light microscopy. In parallel, genomic DNA was directly extracted from faecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was eluted in 100 µl of elution buffer (Qiagen) and stored at −20 °C until use.

Molecular identification of *Blastocystis* sp. and *Cryptosporidium* spp.

Each DNA sample was subjected to the highly sensitive real-time PCR assay based on SYBER-Green I fluorescence developed by Poirier et al. [47], using a pair of *Blastocystis* sp.-specific primers targeting a 320 bp-length domain of the SSU rDNA coding region. For the detection of *Cryptosporidium*, a nested PCR amplifying an 830 bp-length domain of the SSU rRNA gene was performed for each DNA sample, as described previously [48]. The PCR products were sequenced directly on both strands, using the PCR primer pairs. The *Blastocystis* sp. or *Cryptosporidium* spp. SSU rRNA gene sequences obtained were aligned

using the BioEdit v7.0.1 package (http://www.mbio.ncsu.edu:BioEdit/bioedit.html), then compared with the sequences of both parasites published on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/), using the basic local alignment search tool (BLAST) program. *Blastocystis* sp. STs were identified by determining the exact match or closest similarity against all known sequences, according to the updated classification by Alfellani et al. [31]. The SSU rRNA gene sequences obtained in this study were deposited in GenBank under accession numbers KP890047 to KP890053.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) using the Fisher's exact test to explore the relation between the prevalence of gastrointestinal parasites and the stratified age of the dogs (< 1 –year -old vs. ≥ 1 –year -old) or gender. The general significance level was set at a P value below 0.05.

Results

Analysis of the study cohort of dogs

This study focused on 116 household dogs, all living in the Lyons area of France. The mean age of the dogs included in this study was around 37 months (ranging from 1.5 month to 15 years). This canine population included 71 females (61.2%) and 45 males (38.8%) as well as 44 different breeds and 13 cross-breeds.

Prevalence of gastrointestinal parasites

Among the 116 canine faecal samples screened in this study, 40.5% (47 animals) were positive for at least one species of gastrointestinal parasite (Table 1) identified by light microscopy examination of stools. These parasites included the protozoa *Giardia* spp. (29/116) and *Cystoisospora* spp. (23/116), and the helminths *Toxocara canis* (11/116), *Trichuris vulpis* (2/116), *Ancylostoma caninum* (4/116) and *Taenia* spp. (2/116). Monoinfections represented 61.7% of infections, and 18 dogs excreted two to four parasites (Table 1 and additional file). The most common multiple infection was *Giardia* spp. and *Cystoisospora* spp. (12 animals).

Table 1 Prevalence of gastrointestinal parasites in a cohort of dogs overall and stratified by age classes

Parasites	Overall prevalence (n = 116)	Prevalence by age class		P value
		< 1 –year -old (n = 39);	≥ 1 –year -old (n = 77)	
***Giardia* spp.**	25.0% (29)	48.7% (19)	13% (10)	**<0.0001**
***Cystoisospora* spp.**	19.8% (23)	28.2% (11)	15.6% (12)	0.139
***Cryptosporidium* spp.**	2.6% (3)	7.7% (3)	0% (0)	**0.036**
***Blastocystis* spp.**	3.4% (4)	5.1% (2)	2.6% (2)	0.601
Toxocara canis	9.5% (11)	10.3%(4)	9.1% (7)	1.000
Trichuris vulpis	1.7% (2)	0% (0)	2.6% (2)	0.549
Ancylostoma caninum	3.4% (4)	2.6% (1)	3.9% (3)	1.000
***Taenia* spp.**	1.7% (2)	2.6% (1)	1.3% (1)	1.000
Total of infected animals[a]	42.2% (49)	59.0% (23)	33.8% (26)	**0.016**

[a] Animals infected with at least one parasite.

Molecular identification of *Blastocystis* sp. and *Cryptosporidium* spp.

To complete this epidemiological survey, the protozoa *Blastocystis* sp. and *Cryptosporidium* spp. were searched for in canine stool by molecular methods. DNA extracted from the faecal samples was amplified by PCR using *Blastocystis*- and *Cryptosporidium*-specific primer pairs. Of the 116 dogs, only 4 (3.4%) were positive for *Blastocystis* sp. and 3 (2.6%) for *Cryptosporidium* spp. (Tables 1 and 2). By adding these data to those obtained by light microscopy, the overall prevalence of animals infected with at least one gastrointestinal parasite reached 42.2% (49/116). The four dogs infected with *Blastocystis* sp. (Table 2) were of various breeds (crossbreeds of German shepherd and Pyrenean shepherd, basset hound, Yorkshire terrier and English Cocker spaniel), with ages ranging between 2.5 months and 8 years. The sequence analysis of positive PCR products revealed two different *Blastocystis* sp. STs: ST2 in 2 dogs and ST10 in the two others dogs (99 to 100% sequence identity to homologous sequences). Only one animal infected with ST2 presented diarrhea as the only clinical sign of disease, but was also positive for *Giardia* spp. Regarding *Cryptosporidium* spp., the parasite was detected in 3 puppies aged less than 15 -weeks of different breeds (crossbreeds of German shepherd and Newfoundland, Labrador retriever, and beagle). The sequence analysis of the three *Cryptosporidium* spp. isolates identified *C. canis* (100%

identity with homologous sequences available in databases) (Table 1). A mixed infection with *Giardia* spp. was identified in all 3 dogs infected with *Cryptosporidium* spp. and for one dog, presenting diarrhea.

Table 2 Clinical data of dogs infected either by *Blastocystis* sp. or *Cryptosporidium* spp. in the present study

Sample identification	Breed	Age[a]	Sex	Diarrhea	Associated parasite	Anthelmintic treatment[b]	Antiprotozoal treatment[b]	*Blastocystis* sp. ST	*Cryptosporidium* sp.
12-800	Yorkshire terrier	4 y	F	-	-	+	-	ST10	-
12-802	English Cocker spaniel	2.5 m	F	-	*Giardia* spp.	+	-	ST10	-
12-944	German shepherd x Pyrenean shepherd	8 y	M	-	-	+	-	ST2	-
13-128	Basset hound	8 m	M	+	*Giardia* spp.	+	+	ST2	-
13-442	Labrador retriever	2 m	M	+	*Giardia* spp. *Cystoisospora* spp.	+	-	-	*C. canis*
13-453	German shepherd x Newfoundland	3.5 m	F	-	*Giardia* spp.	+	-	-	*C. canis*
13-457	Beagle	3.5 m	F	-	*Giardia* spp.	+	-	-	*C. canis*

[a] y: years; m: months.

[b] Within 6 months prior to sampling.

Differences in the distribution of parasite infections

In this canine population, there was no significant difference in the distribution of parasite infections between male and female dogs ($P > 0.05$). Total infection of the gastrointestinal parasites was significantly higher ($P = 0.016$; odds ratio (OR): 2.82; 95% confidence interval (CI): 1.28 - 6.24) in < 1 –year -old dogs (59.0%) than in ≥ 1 –year -old dogs (33.8%) (Table 1). In addition, there were significant differences in the prevalence of *Giardia* spp. (48.7% vs. 13%; $P < 0.0001$; OR = 6.36; 95% CI 2.55 – 15.88) and *Cryptosporidium* spp. (7.7% vs. 0%; $P = 0.036$; OR = 3.13; 95% CI, 0.74 – 295.5) between age classes (Table 1).

Discussion

The overall prevalence of canine intestinal parasites identified in this study, using both stool light microscopy examination and molecular methods, revealed a high level of infection: 42.2% of the household dogs analyzed presented at least one gastrointestinal parasite. The comparison of this prevalence with that obtained in previous studies on household dog populations remains difficult due to differences in the sensitivity of laboratory techniques, the experience of the observers performing the analysis, and the characteristics of the canine groups investigated. However, this prevalence is roughly similar to that observed for instance, in Belgium [7], Brazil [6], Argentina [3], and Canada [13]. Although this value is high in household dogs, the prevalence of gastrointestinal parasitism may be comparatively even higher in populations of puppies, shelter-resident dogs, stray dogs, breeding kennel dogs, and sled dogs in relation to the age of the animals, population density, inadequate sanitary conditions, or lack of veterinary care [6,7,9,14,16]. In numerous studies performed on household dogs, including our survey, *Giardia* spp. was the most common intestinal parasite [7,13], and like various other parasites is significantly more prevalent in younger dogs than in older dogs [4,5,7,13].

Regarding *Cryptosporidium* spp., the prevalence observed in the present study is very low: only 2.6% (3/116) of the dogs were infected with this parasite. This prevalence is close to that reported in canine populations, for instance in Italy [49], Thailand [50], Brazil [6,28], the Netherlands [8], or China [22]. On the other hand, it is much lower than the 25.9% prevalence recently observed in a study conducted in puppies housed in breeding kennels in France [11]. Such a difference between these two French studies might be explained in particular by significant variations in the age of the animals and population density, as overcrowding in kennels naturally promotes parasite transmission. In that sense, only puppies aged less than 14 weeks were infected with *Cryptosporidium* spp. in our survey, and the infection with this parasite was significantly correlated with the age of the dogs. All of this data confirms that the prevalence of canine cryptosporidiosis is dependent on host age, in agreement with previous studies [11,13,19,21,51,52]. At the molecular level, the three *Cryptosporidium* spp. isolates found in this study were identified as *C. canis*. As already stated by others [17,21], the majority of infections in dogs are effectively caused by *C. canis*. This species has also been shown to infect humans, notably children and immunocompromised patients in developing countries [21,53,54]. However, the rate of infection by *C. canis* is extremely low, and most

cases of human cryptosporidiosis worldwide are associated with *C. hominis* and *C. parvum* [17,19,55-57]. Therefore, according to the low prevalence of *Cryptosporidium* spp. in the household dog population in France and the narrow host range of *C. canis*, our data strengthen the hypothesis that this parasite is very likely of minimal zoonotic risk. To reinforce this statement, the identification of mixed infection with *C. hominis* in HIV patients may reflect anthroponotic rather than zoonotic transmission of *C. canis* [58]. Consequently, *C. canis* would have limited public health significance in the general population [17,21,23,59], even though the parasite might be a concern when pets are living in contact with immunosuppressed owners. Based on our present knowledge, most dogs infected with *Cryptosporidium* spp. are asymptomatic [20]. This was confirmed in our study, since two dogs infected with *C. canis* were healthy carriers. Interestingly, the third infected dog presented diarrhea, a common clinical sign of cryptosporidiosis. However, this animal was also infected with *Giardia* spp., another potential cause of diarrhea [60]. Consequently, even though the number of *C. canis*-infected dogs was limited, no association between *C. canis* infection and gastrointestinal symptoms could be clearly established from our data.

Regarding *Blastocystis* sp., only four dogs (3.4%) were found to be infected by this parasite in our canine population. This very low prevalence was comparable to that reported among pets and pound dogs in Australia (2.5%), and semi-domesticated dogs in Cambodia (1.3%) [Wang et al. 2013]. In the same order of value, 54 faecal samples obtained from dogs housed in an animal shelter in Japan were completely free of *Blastocystis* sp. after microscopic examination [61]. Identical results were obtained by analyzing stools from 30 privately owned and 42 stray dogs in Greece [62], 20 household dogs in Germany [63], 56 farm and rural dogs in Australia [Roberts et al. 2013], and 49 rural dogs in Brazil [28]. However, in further studies mainly focusing on either, stray or shelter dog populations, the prevalence of *Blastocystis* sp. was significantly higher, reaching 24% in India [46], 28% in Iran [41], 37% in Colombia [27], and 70% in Australia [64]. In view of this data, stray dogs living mostly in rural areas or densely populated cities with poor sanitation and hygiene were recognized to be at higher risk for carrying *Blastocystis* sp. than household dogs, as confirmed in our survey. In this regard, Ruaux and Stang [44] also showed that the prevalence of *Blastocystis* sp. was zero in a population of client-owned domestic dogs, and reached 10% in shelter-resident dogs, with both canine populations living in the same area of Oregon state (USA). Moreover, as in previous studies [41,64], no correlation was found in our survey between the presence of *Blastocystis* sp. and either the age or gender of the host.

Strikingly, a large diversity of *Blastocystis* sp. STs was previously identified in faecal samples of various canine populations including stray, shelter-resident and household dogs. This included ST1 [33,42-44,46], ST2 [27,33], ST3 [36,43], ST4 [43,46], ST5 [46], ST6 [46], ST7 [42], and ST10 [44]. Alfellani et al. [32], in a review of the relative global distribution of *Blastocystis* sp. STs identified in humans worldwide, found that ST3 is largely predominant, followed by ST1, ST2, and ST4. The other STs (ST5 to ST9) are episodically found in humans, and are likely the result of zoonotic transmission. Indeed, ST5 frequently infects pigs [34,40], while ST6 and ST7 are conceived as avian STs and are quite rare in other hosts [31]. In our study, we identified ST2 and ST10, as already found in dogs as stated above. ST2 is common in humans, but also in various groups of animals, including primates [31]. In the case of ST10, it has never been found in humans, but is frequently identified in bovines [31]. Based on both our own and other research data, predominant or specific- STs were not identified in the overall canine population.

Consequently, the search of the origin of *Blastocystis* sp. isolates in dogs, together with their zoonotic potential, remains the subject of much discussion. A recent study has demonstrated that domestic dogs in close contact with patients infected with *Blastocystis* sp. harbored ST1 and ST7 in common with their owners [42], suggesting that the source of parasite infection of the owners might be their household dogs. However, in the same study, the presence of both STs, including the avian ST7, were also identified in pet birds owned by the patients. Moreover, some tap water samples collected from the patients' houses were also contaminated with *Blastocystis* sp. ST1. Therefore, in our opinion, the source of transmission of *Blastocystis* sp. to humans was not clearly determined and could be zoonotic, environmental, or simply anthroponotic. In a second study, Nagel et al. [43] found that 8 dogs in close contact with 11 symptomatic family members infected by *Blastocystis* sp. were all positive for the parasite, and harbored at least one ST in common with each of the corresponding patients. This consistency in the STs frequently found in humans between these two hosts still did not prove zoonotic transmission of the parasite, since the contamination of dogs by humans, environmental, or other animal sources should not be excluded. Additionally, identical alleles of ST1, ST2, and ST3 in humans and animals including dogs but also rats and cattle, were identified in an epidemiological study performed in Colombia [27]. Even if dogs could potentially contribute to transmission of the parasite to humans, the proposed primary source of infection was water supplies contaminated by cattle

feces. A survey performed in a rural community in Nepal including humans and various farm animals reared by the villagers, demonstrated that drinking water contaminated by feces of both humans and animals facilitated waterborne transmission [39]. Therefore, considering these observations in addition to those of our survey, combined with the low global prevalence of the parasite in the canine population, and the absence of dog-specific/predominant ST, these pets are unlikely to be natural hosts of *Blastocystis* sp. as recently suggested [44,46], and potentially opportunistically infected by other host faeces or contaminated after ingestion of drinking water or food.

The question of the pathogenicity of *Blastocystis* sp. in dogs (as in other animal groups) remains open, due to insufficient clinical and epidemiological data. From the data reported in a recent study, it appears unlikely that the presence of *Blastocystis* sp. infection is a significant contributor to gastrointestinal signs in a canine population [44]. In a clinical case report on the presence of numerous *Blastocystis* sp. cells after cytological examination of a rectal scrapping from a 3 –year-old dog with diarrhea, the absence of the parasite or gastrointestinal lesions was described on endoscopic biopsies collected from the stomach, duodenum, ileum, and colon of the animal [65]. These observations indicate that *Blastocystis* sp. is not likely a major factor in the development of diarrhea or other clinical signs, and that alteration of gastrointestinal flora secondary to the underlying pancreatic disease allowed overgrowth of the parasite. Another epidemiological study performed in a population of dogs with diarrhea in Chile showed a high prevalence of *Blastocystis* sp. in fecal samples [4]. However, the animals were also infected with various parasites, including *Giardia* spp., *Amoeba* sp., *Cystoisospora* spp., and helminths, which are all potentially responsible for gastrointestinal symptoms. In our study, only one dog infected with *Blastocystis* sp. ST2 presented diarrhea, but was also infected with *Giardia* spp. Consequently, no association between *Blastocystis* sp. infection and gastrointestinal symptoms can be clearly established from our data as well as the data found in the literature.

Conclusions

To our knowledge, this study presents the first molecular epidemiological data regarding the zoonotic parasites *Cryptosporidium* spp. and *Blastocystis* sp. in a population of domestic dogs in France. Overall, the observed prevalence of both parasites (3.4 and 2.6%, respectively) was extremely low, and likely reflected the good sanitary and hygiene conditions of these

household animals, a high proportion of which were indeed owned by veterinary students. Only the species *C. canis* episodically found in humans was identified in our canine population, thus confirming the limited risk of zoonotic transmission of this parasite. Moreover, the low prevalence of *Blastocystis* sp. in the canine population, combined with the absence of dog-specific/predominant ST, strongly suggests that the parasite infection is transient in household dogs. In our opinion, these animals were occasionally infected through exposure to multiple sources, including faeces of other animal hosts, or contaminated food or drinking water in their immediate environment. Therefore, these dogs are unlikely to represent a potential source of zoonotic infection to their owners. More intensive sampling worldwide should be performed in order to confirm the low impact of both parasites in the canine population.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

EV LZ and GC conceived the study and designed the experiments. MO JB DES MTP NG and MH collected the samples and/or performed the experiments. SBV LD and MH performed general supervision tasks. EV GC SBV MH and LZ participated in the interpretation of data. EV GC and MO wrote the manuscript. All authors have read and approved the final version of the manuscript.

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3. Prévalence et caractérisation moléculaire de *Cryptosporidium* chez plusieurs groupes d'animaux des parcs zoologiques français

Préambule : Ce travail fait partie d'un projet de mon laboratoire d'accueil Français qui a pour but principal l'étude de la prévalence et de la diversité génétique des protistes *Blastocystis* sp. et *Cryptosporidium* spp. dans différentes populations d'animaux des parcs zoologiques français. En tant que doctorant, je me suis particulièrement impliqué dans l'identification de *Cryptosporidium.* Ce qui vous ait présenté ci-dessous sont les résultats préleminaires de ce travail.

« Prévalence et caractérisation moléculaire de *Cryptosporidium* chez plusieurs groupes d'animaux des parcs zoologiques français ».

Introduction :

En ce qui concerne ce protiste intestinal, il a été rapporté que ce parasite peut infecter plus de 150 espèces de mammifères et son potentiel zoonotique a maintes fois été rapporté (Ryan, Fayer, and Xiao, 2014; Xiao, 2010). C'est pourquoi, une meilleure connaissance de l'épidémiologie de cette parasitose chez les animaux constitue un élément clef pour contrôler et limiter sa transmission à l'homme. Plusieurs études ont déjà été réalisées chez différentes espèces d'animaux sauvages ou en captivité : primates, pandas, serpents, oiseaux, tortues, etc. afin de mettre en évidence de potentiels réservoirs de la cryptosporidiose (Cerveny et al., 2012; Gibbons and Steffes, 2013; Sak et al., 2014; Schulze et al., 2012; Wang et al., 2015). Ce parasite a notamment été recherché dans plusieurs zoos européens (Gomez et al., 2000; Gracenea et al., 2002; Levecke et al., 2007) mais jamais en France. C'est pourquoi, nous avons cherché à déterminer la prévalence et la nature de la diversité génétique de *Cryptosporidium* spp. chez plusieurs populations animales en captivité dans deux zoos Français, le zoo de la Palmyre qui se situe dans le sud-ouest de la Charente-Maritime et le zoo de Lille qui se situe à Lille, au nord de la France.

Matériels et méthodes :

a) Déclaration éthique :

La permission pour cette étude a été obtenue par les vétérinaires directeurs des 2 parcs zoologiques. L'étude présente était conformément à la Convention européenne pour la Protection d'Animaux Vertébrés utilisés pour des Buts Scientifiques Expérimentaux et Autres.

b) Constitution de la collection de selles :

Pour réaliser la collecte des selles, deux campagnes ont été organisées : une première de deux jours au parc zoologique de la Palmyre situé dans le sud-ouest de la Charente-Maritime en avril 2013 (Figure 1) et une seconde d'un jour au parc zoologique de Lille en Juin 2014 (Figure 2). Au total, 305 échantillons ont été collectés (207 à la Palmyre et 98 à Lille). Les prélèvements de selles ont collectés dans les enclos des animaux très tôt le matin. Pour la collecte les critères suivants ont été respectés : 1. Utilisation d'un pair de gants de latex par échantillon. 2. Utilisation des pots stériles par échantillon. 3. Collection d'au moins 5 g de fèces par échantillon. Certains échantillons correspondaient à des groupes d'individus de la même espèce (pool de selles) et d'autres plus rares ont pu être obtenus à partir d'un seul individu (selles individualisées). Les échantillons ont été conservés à -20°C. Des formulaires avec des informations relatives à l'identification des animaux, l'âge, le sexe, etc. ont été complétés. Les espèces animales analysées appartenant à différents groupes taxonomiques sont répertoriées dans le tableau 1.

Figure 1: Plan du parc zoologique de La Palmyre et description et localisation des différents groupes d'animaux hébergés. (http://www.voyagemag.fr/dossiers/parcs-zoologiques/zoo-de-la-palmyre/)

Figure 2: Plan du parc zoologique de Lille et description et localisation des différents groupes d'animaux hébergés. (http://www.lille.fr/cms/accueil/sport-loisirs/zoo-lille/infos-pratiques-zoo/plan-zoo-lille)

b) Extraction d'ADN à partir des selles et PCR nichée :

L'ADN total des selles (0.18 à 0.2 g/échantillon) a été extrait à l'aide du Mini Kit QIAamp DNA stool (Qiagen®) suivant les instructions du fabricant et après une étape de lyse mécanique réalisée pendant 5 minutes à l'aide de billes en verres (Sigma-Aldrich®, 425-600 µm). Les ADN obtenus ont été conservés à -20°C jusqu'à leur utilisation.

Pour la détection du parasite et l'étude génotypique des prélèvements fécaux, une PCR nichée ciblant l'ADNr 18S a été utilisée. L'ADN amplifié correspond à un fragment entre 820 et 864 pb du gène codant pour la sous unité 18S de l'ARN ribosomal de *Cryptosporidium* (Xiao et al, 1999). Les amorces utilisées dans ce travail sont présentées dans le Tableau 2.

Tableau 1. Distribution des échantillons issus de différentes hôtes animaux des deux zoos français: Zoo de la Palmyre et Zoo de Lille.

Nom Familier	Nom Scientifique	Zoo	Nombre d'échantillons[a]	Echantillon positif pour *Cryptosporidium*
EUTHERIA (PLACENTAL MAMMALS)				
Primates				
Catarrhini				
Hominidae				
Western gorilla	*Gorilla gorilla*	La Palmyre	6	0
Orangutan	*Pongo pygmaeus*	La Palmyre	3	0
Chimpanzee	*Pan troglodytes*	La Palmyre	3	0
Hylobatidae				
Lar gibbon	*Hylobates lar*	Lille	2	0
Lar gibbon	*Hylobates lar*	La Palmyre	1	0
Buff-cheeked gibbon	*Nomascus gabriellae*	La Palmyre	2	0
Siamang	*Symphalangus syndactylus*	Lille	4	0
Cercopithecidae				
Southern pig-tailed macaque	*Macaca nemestrina*	La Palmyre	3	0
Mandrill	*Mandrillus sphinx*	La Palmyre	1	0
Owl-faced monkey	*Cercopithecus hamlyni*	La Palmyre	2	0
Roloway monkey	*Cercopithecus roloway*	La Palmyre	1	0

L'hoest's monkey	*Cercopithecus lhoesti*	La Palmyre	1	0
De Brazza's monkey	*Cercopithecus neglectus*	La Palmyre	2	0
Eastern black-and-white colobus	*Colobus guereza*	La Palmyre	3	0
Platyrrhini				
Cebidae[b]				
Brown capuchin	*Cebus apella*	Lille	1	0
Golden-bellied capuchin	*Cebus xanthosternos*	La Palmyre	2	0
Common squirrel monkey	*Saimiri sciureus*	La Palmyre	1	0
Emperor tamarin	*Saguinus imperator*	Lille	1	0
Emperor tamarin	*Saguinus imperator*	La Palmyre	2	0
White-lipped tamarin	*Saguinus labiatus*	Lille	1	0
Red-handed tamarin	*Saguinus midas*	La Palmyre	2	0
Pied tamarin	*Saguinus bicolor*	La Palmyre	1	0
Cotton-top tamarin	*Saguinus oedipus*	La Palmyre	1	0
Golden-headed lion tamarin	*Leontopithecus chrysomelas*	La Palmyre	4	0
Golden lion tamarin	*Leontopithecus rosalia*	La Palmyre	1	0
Geoffroys's marmoset	*Callithrix geoffroyi*	Lille	1	0
Geoffroys's marmoset	*Callithrix geoffroyi*	La Palmyre	1	0
Pigmy marmoset	*Callithrix pygmaea*	La Palmyre	2	0
Common marmoset	*Callithrix jacchus*	La Palmyre	1	0
Goeldi's marmoset	*Callimico goeldii*	La Palmyre	2	0

Pitheciidae				
White-faced saki	*Pithecia pithecia*	Lille	1	0
Strepsirrhini				
Lemuridae				
Ring-tailed lemur	*Lemur catta*	Lille	1	0
Ring-tailed lemur	*Lemur catta*	La Palmyre	4	0
Red ruffed lemur	*Varecia rubra*	Lille	1	0
Red ruffed lemur	*Varecia rubra*	La Palmyre	2	0
Black-and-white ruffed lemur	*Varecia variegata*	Lille	1	0
Black-and-white ruffed lemur	*Varecia variegata*	La Palmyre	3	0
Black lemur	*Eulemur macaco*	La Palmyre	1	0
Lorisidae				
Pygmy slow loris	*Nycticebus pygmaeus*	Lille	1	0
Carnivora				
Feliformia				
Felidae				
Northern lynx	*Lynx lynx*	La Palmyre	1	0
Lion	*Panthera leo*	La Palmyre	1	0
Cheetah	*Acinonyx jubatus*	La Palmyre	5	0
Leopard	*Panthera pardus*	La Palmyre	2	0
Snow leopard	*Panthera uncia*	La Palmyre	2	0
Jaguar	*Panthera onca*	La Palmyre	2	0

Tiger	*Panthera tigris*	La Palmyre	2	0
Viverridae				
Binturong	*Arctictis binturong*	Lille	1	0
Herpestidae				
Yellow mongoose	*Cynictis penicillata*	Lille	1	0
Slender-tailed meerkat	*Suricata suricatta*	Lille	1	0
Slender-tailed meerkat	*Suricata suricatta*	La Palmyre	2	0
Caniformia				
Canidae				
Grey wolf	*Canis lupus*	La Palmyre	4	0
African hunting dog	*Lycaon pictus*	La Palmyre	3	0
Fennec fox	*Vulpes zerda*	La Palmyre	7	0
Ailuridae				
Red panda	*Ailurus fulgens*	Lille	2	0
Red panda	*Ailurus fulgens*	La Palmyre	2	0
Ursidae				
Polar bear	*Ursus maritimus*	La Palmyre	3	0
Mustelidae				
Oriental small-clawed otter	*Aonyx cinerea*	La Palmyre	1	0
Procyonidae				
Kinkajou	*Potos flavus*	Lille	1	0
Brown-nosed coati	*Nasua nasua*	Lille	1	0

Brown-nosed coati	*Nasua nasua*	La Palmyre	3	0
Otariidae				
California sea lion	*Zalophus californianus*	La Palmyre	6	0
Artiodactyla				
Camelidae				
Alpaca	*Vicugna pacos*	Lille	1	0
Alpaca	*Vicugna pacos*	La Palmyre	5	0
Hippopotamidae				
Hippopotamus	*Hippopotamus amphibius*	La Palmyre	1	0
Giraffidae				
Giraffe	*Giraffa camelopardalis*	La Palmyre	6	0
Tragulidae				
Java mouse-deer	*Tragulus javanicus*	Lille	1	0
Bovidae				
Common eland	*Taurotragus oryx*	Lille	1	0
Greater kudu	*Tragelaphus strepsiceros*	La Palmyre	2	0
Bongo	*Tragelaphus eurycerus*	La Palmyre	1	0
American bison	*Bison bison*	La Palmyre	3	0
Blinded wildebeest	*Connochaetes taurinus*	La Palmyre	1	0
Bontebok	*Damaliscus pygargus*	La Palmyre	1	0
Beisa oryx	*Oryx beisa*	La Palmyre	4	0
Scimitar-horned oryx	*Oryx dammah*	La Palmyre	5	0

Goat	*Capra hircus*	La Palmyre	7	0
Impala	*Aepyceros melampus*	La Palmyre	1	0
Perissodactyla				
Equidae				
Common zebra	*Equus burchellii*	Lille	2	0
Common zebra	*Equus burchellii*	La Palmyre	2	0
Grevy's zebra	*Equus grevyi*	La Palmyre	2	0
African wild ass	*Equus asinus*	La Palmyre	2	0
Rhinocerotidae				
White rhinoceros	*Ceratotherium simum*	Lille	2	0
White rhinoceros	*Ceratotherium simum*	La Palmyre	1	0
Tapiridae				
South American tapir	*Tapirus terrestris*	Lille	1	0
South American tapir	*Tapirus terrestris*	La Palmyre	2	0
Proboscidea				
Asiatic elephant	*Elephas maximus*	La Palmyre	4	0
Rodentia				
House mouse[b]	*Mus musculus*	Lille	2	0
Norway rat[b]	*Rattus norvegicus*	Lille	2	0
Indian crested porcupine	*Hystrix indica*	Lille	2	0
Patagonian mara	*Dolichotis patagonum*	Lille	3	0
Capybara	*Hydrochoerus hydrochaeris*	Lille	2	0

Capybara	*Hydrochoerus hydrochaeris*	La Palmyre	3	0
Chiroptera				
Lyle's flying fox	*Pteropus lylei*	Lille	2	0
Rodrigues flying fox	*Pteropus rodricensis*	La Palmyre	1	0
Egyptian rousette	*Rousettus aegyptiacus*	La Palmyre	1	0
MARSUPIALIA				
Red kangoroo	*Macropus rufus*	La Palmyre	1	0
Red-necked wallaby	*Macropus rufogriseus*	La Palmyre	1	0
AVES				
Galliformes				
Crested wood partridge	*Rollulus rouloul*	Lille	1	0
Common peafowl	*Pavo cristatus*	La Palmyre	2	0
Anseriformes				
Bar-headed goose	*Anser indicus*	Lille	1	0
Bar-headed goose	*Anser indicus*	La Palmyre	1	0
Barnacle goose	*Branta leucopsis*	Lille	1	0
Nene	*Branta sandvicensis*	Lille	2	0
Mandarin duck	*Aix galericulata*	Lille	1	0
Tufted duck	*Aythya fuligula*	Lille	1	0
Ferriginous duck	*Aythya nyroca*	Lille	3	0

Hottentot teal	*Anas hottentota*	Lille	1	0
Black swan	*Cygnus atratus*	La Palmyre	1	0
Psittaciformes				
Twenty-eight parrot	*Barnardius zonarius*	Lille	1	0
Grey parrot	*Psittacus erithacus*	Lille	1	0
Senegal parrot	*Poicephalus senegalus*	Lille	1	0
Burrowing parrot	*Cyanoliseus patagonus*	Lille	1	0
Green-winged macaw	*Ara chloroptera*	Lille	1	0
Green-winged macaw	*Ara chloroptera*	La Palmyre	4	0
Buffon's macaw	*Ara ambigua*	La Palmyre	1	0
Scarlet macaw	*Ara macao*	Lille	1	0
Blue-and-yellow macaw	*Ara ararauna*	La Palmyre	4	0
Hyacinth macaw	*Anodorhynchus hyacinthinus*	La Palmyre	1	0
Blue-crowned conure	*Aratinga acuticaudata*	Lille	1	0
Monk parakeet	*Myiopsitta monachus*	Lille	1	0
Orange-winged amazon	*Amazona amazonica*	Lille	1	0
Mealy amazon	*Amazona farinosa*	Lille	1	0
Yellow-crowned amazon	*Amazona ochrocephala*	Lille	1	0
Rosella	*Platycercus eximus*	La Palmyre	1	**1**
Lesser sulphur-crested cockatoo	*Cacatua sulphurea*	La Palmyre	1	0
Strigiformes				
Snowy owl	*Bubo scandiacus*	Lille	1	0

Coraciiformes				
Laughing kookaburra	*Dacelo novaeguineae*	Lille	2	0
Passeriformes				
Javan sparrow	*Lonchura oryzivora*	Lille	1	0
Phoenicopteriformes				
Chilean flamengo	*Phoenicopterus chilensis*	La Palmyre	6	0
American flamengo	*Phoenicopterus ruber*	La Palmyre	3	0
Pelecaliformes				
Scarlet ibis	*Eudocimus ruber*	La Palmyre	4	0
Accipitriformes				
Rüppel's griffon vulture	*Gyps rueppellii*	La Palmyre	1	0
Ciconiiformes				
Marabou stork	*Leptoptilos crumeniferus*	La Palmyre	1	0
Columbiformes				
Nicobar pigeon	*Caloenas nicobarica*	La Palmyre	1	0
Bucerotiformes				
Southern ground-hornbill	*Bucorvus leadbeateri*	La Palmyre	1	0
Trumpeter hornbill	*Bycanistes bucinator*	La Palmyre	1	0
Great Indian hornbill	*Buceros bicornis*	La Palmyre	1	0
Passeriformes				
Bali mynah	*Leucopsar rotschildi*	La Palmyre	1	0

Gruiformes				
Black crowned-crane	*Balearica pavonina*	La Palmyre	2	0
Sphenisciformes				
Jackass penguin	*Spheniscus demersus*	La Palmyre	1	0
Ratites				
Common ostrich	*Struthio camelus*	La Palmyre	2	**1**
Greater rhea	*Rhea americana*	La Palmyre	3	0
CROCODYLIA				
African slender-snouted crocodile	*Mecistops cataphractus*	La Palmyre	1	0
SQUAMATA				
Green iguana	*Iguana iguana*	Lille	1	0
Green iguana	*Iguana iguana*	La Palmyre	1	0
Green anaconda	*Eunectes murinus*	La Palmyre	1	0
Boa constrictor	*Boa constrictor*	Lille	1	0
Cornsnake	*Pantherophis guttatus*	Lille	2	0
Russian ratsnake	*Elaphe schrencki*	Lille	1	0
Taiwan beauty snake	*Elaphe taeniura*	Lille	1	0
Common kingsnake	*Lampropeltis getula*	Lille	2	**1**
Milksnake	*Lampropeltis triangulum*	Lille	2	0

TESTUDINES				
Aldabra tortoise	*Aldabrachelys gigantea*	Lille	1	0
Aldabra tortoise	*Aldabrachelys gigantea*	La Palmyre	1	0
Spur-thighed tortoise	*Testudo graeca*	Lille	1	0
INSECTA[c]				
Madagascar hissing cockroach	*Gromphadorhina portentosa*	Lille	3	0
Giant cockroach	*Blaberus giganteus*	Lille	1	0
Peppered roach	*Archimandrita tessellata*	Lille	1	0
Dubia roach	*Blaptica dubia*	Lille	3	0
Sun beetle	*Pachnoda marginata*	Lille	2	0
Desert locus	*Schistocerca gregaria*	Lille	2	0
Field cricket	*Gryllus bimaculatus*	Lille	5	0

[a] Dépend de l'espèce, les échantillons ont été obtenus à partir de groupes d'individus de la même espèce (mélanges de selles) et d'autres plus rares ont pu être obtenus à partir d'un seul individu (selles individualisées). Pour les insectes, le système digestif a été extrait et analysé après dissection de la population (échantillon individualisé).

[b] Selon Groves (2005), le Cebidae comprend l'ancienne famille de Callitrichidae (ouistitis et tamarins)

[c] Animaux élevés dans le zoo de Lille et utilisés comme aliments pour les autres animaux

Tableau 2. Liste des amorces utilisées pour la réalisation de la PCR nichée

Oligonucléotide	**Utilisation**	**Séquence (5'3')**	**Tm**
18SXIAOEXTF	primer PCR 1 (+)	TTC TAG AGC TAA TAC ATG CG	46.6
18SXIAOEXTR	primer PCR 1 (-)	CCC ATT TCC TTC GAA ACA GGA	50.1
18SXIAOINTF	primer PCR 2 (+)	GGA AGG GTT GTA TTT ATT AGA TAA AG	49.7
18SXIAOINTR	primer PCR 2 (-)	AAG GAG TAA GGA ACA ACC TCC A	50.8

Dans la première étape de PCR, le mélange réactionnel contient 5 µl de tampon de PCR 10X à une concentration finale de 1X, 8 µl de MgCl2 à une concentration finale de 4 mM, 4 µl de dNTPs à une concentration finale de 200 µM, 0.5 µl de chaque amorce externe à une concentration finale de 0.1 µM, 0.3 µl de Hot Start Taq DNA Polymerase Qiagen® (1.5 unités), 10 µl d'ADN pur ou dilué au 1/10ème et 1/100ème, tout pour un volume final de 50 µl. L'amplification d'ADN est menée sur un thermocycleur (MasterCycler EP,Eppendorf). Le programme comprend 10 min de dénaturation à 94°C, suivi de 40 cycles comprenant chacun une étape de dénaturation de 45s à 94 °C, une phase d'hybridation de 45s à 65°C, et une période d'extension de 1 min à 72°C. Le programme se termine par une dernière étape d'extension de 5 min à 72°C.

Pour la seconde étape de PCR, le mélange réactionnel contient 5 µl de tampon de PCR 10X à une concentration finale de 1X, 10 µl de MgCl2 à une concentration finale de 5 mM, 4 µl de dNTPs à une concentration finale de 200 µM, 0.5 µl de chaque amorce externe à une concentration finale de 0.1 µM, 0.25 µl de Hot Start Taq DNA Polymerase Qiagen® (1.5 unités), 2 µl d'ADN de la 1ère PCR, tout pour un volume final de 50 µl. L'amplification d'ADN est faite suivant les mêmes cycles thermiques que la première PCR.

Afin de détecter la présence d'inhibiteurs de la Taq Polymérase, un témoin interne d'amplification, avec un ADN connu pour *Cryptosporidium* (1µl), a été testé sur des extraits d'ADN trouvés négatifs pour *Cryptosporidium* spp.

Une électrophorèse sur gel d'agarose à 2% en tampon TBE 1X (Quantum Biotechnologies) contenant 0.5 µg/ml de bromure d'éthidium sous voltage de 100-120 volts est réalisée pour observer le fragment d'ADN amplifié.

Les produits de PCR positifs ont été ensuite purifiés puis séquencés. Afin de déterminer les espèces de *Cryptosporidium* responsables de l'infection, les séquences des isolats de *Cryptosporidium* spp. obtenues ont été comparées à celles d'isolats de *Cryptosporidium* spp. disponibles dans la base de données du National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Résultats :

Cent quarante espèces animales différentes ont été analysées pour la présence de *Cryptosporidium* spp. (Tableau 1). Trois échantillons ont été détectés positifs par PCR nichée et leurs séquences correspondantes présentent une similarité supérieur à 99% avec celles des espèces connues.

- Au parc zoologique de la Palmyre :

Cryptosporidium a été mis en évidence dans deux échantillons. Il s'agit de selles prélevées à partir de deux espèces d'oiseaux : le Perruche omnicolore (*Platycercus eximus*) et l'Autruche d'Afrique (*Struthio camelus*). Ce qui représente une prévalence de 4.6% chez le groupe d'oiseaux et de 1% dans toute la population animale analysée. Le séquençage des produits de PCR des isolats détectés a permis d'identifier deux espèces de *Cryptosporidium*: *C. galli* pour la première espèce d'oiseau et *C. andersoni* pour la seconde.

- Au parc zoologique de Lille :

Un seul échantillons s'est avéré infecté par *Cryptosporidium* spp.. Celui-çi correspond aux selles prélevées à partir d'un reptile : le serpent *Lampropeltis getula* et l'espèce *C. tyzzeri* a pu être identifiée. Ce qui représente une prévalence de 10% chez les squamates et de 1% de toute la population animale analysée.

Ces résultats seront discutés dans la section « Discussion »

4. Article 5 :

Titre: « Identification of *Cryptosporidium* species in fish from Lake Geneva (Lac Léman) in France ».

Préambule : Cette étude a fait l'objet d'un article publié dans le journal Plos One (sous press).

Résumé :

Cette recherche a été conduite dans le cadre du projet Fish-Parasites (ANR-ALIA 2010) coordonné par mon laboratoire d'accueil. L'objectif principal de cette action a été d'améliorer la sécurité sanitaire des produits de la pêche en identifiant le risque parasitaire et en automatisant la détection des parasites en France. Je me suis impliqué particulièrement dans l'identification de *Cryptosporidium*.

Cryptosporidium, protiste protozoaire capable de causer des diarrhées sévères chez un large nombre de vertébrés y compris l'homme, est reconnu de plus en plus comme un parasite des espèces animales sauvages. Cependant, peu de données sont disponibles concernant l'identification des espèces et des génotypes de *Cryptosporidium* dans les milieux aquatiques, et plus particulièrement dans les poissons d'eau douce comestibles. C'est pourquoi, il était intéressant d'évaluer la prévalence de *Cryptosporidium* spp. ainsi que d'identifier de nouveaux hôtes pour ce protiste chez les poissons d'eau douce provenant du Lac Léman (lac de Genève) en France, important réservoir d'eau potable de sa région.

Pour mener ectte étude, ce parasite a été recherché dans les muqueuses intestinales et stomacales de 42 poissons d'eau douce provenant du Lac Léman. Au total, 41 poissons entiers et 100 filets (coupures de chair de poisson) ont été recueillis auprès de fournisseurs de la pêche autour du lac. Une extraction de l'ADN total a été réalisée en utilisant le kit NucleoSpin™ (Macherey-Nagel, GmbH & Co KG, Germany) suivie d'une PCR nichée utilisant des amorces dégénérées. Les produits de PCR ont été ensuite purifiés puis séquencés et analysés. Le polymorphisme génétique des isolats de *C. parvum* a été étudié à l'aide du marqueur moléculaire gp60. Les principaux résultats sont listés ci-dessous:

1. Cinq espèces de poissons ont été identifiées comme des hôtes potentiels de *Cryptosporidium*: *Salvelinus alpinus*, *Esox lucius*, *Coregonus lavaretus*, *Perca fluviatilis*, and *Rutilus rutilus*.

2. La présence de *Cryptosporidium* spp. a été retrouvée chez 15 des 41 poissons (37%), répartis comme suit: 13 (87%) *C. parvum*, 1 (7%) *C. molnari*, et 1 (7%) infection mixte (*C. parvum* et *C. molnari*). *C. molnari* a été identifié dans l'estomac, tandis que *C. parvum* a été trouvé dans l'estomac et l'intestin.

3. Afin d'identifier les sous-types de *Cryptosporidium*, une amplification du fragment d'ADN codant la glycoproteine de 60 kDa (gp60) suivi d'un séquençage de l'amplicon ont été réalisés. Parmi les échantillons inféctés par *C. parvum*, trois sous-types ont été identifiés: IIaA15G2R1, IIaA16G2R1 et IIaA17G2R1. L'examen histologique a confirmé la présence de *C. parvum* dans les cellules épithéliales digestives.

4. L'examen microscopique des tissus de poissons d'eau douce infectés par *C. parvum* a révélé la présence de différents stades évolutifs du parasite au niveau de la bordure apicale de l'épithélium gastrique et intestinal ce qui plaiderait plutôt en faveur d'une réelle infection du poisson plutôt que d'un simple portage.

5. Afin de déterminer si les filets de poissons pouvaient être contaminés par *Cryptosporidium* provenant du tube digestif, les filets de 100 perches (*Perca fluviatilis*) ont été analysés et la présence de *C. molnari* a été confirmée dans les filets d'un seul individu.

En conclusion cette étude a permis de rapporter les premières données d'épidémiologie moléculaire sur *Cryptosporidium* spp. chez les poissons en France. Une forte prévalence de cryptosporidiose a été identifiée avec une prédominance de l'espèce *C. parvum* chez les poissons d'eau douce. Trois sous-types zoonotiques de *C. parvum* largement décrits dans le monde ont été identifiés présageant que les poissons peuvent être une source potentielle d'infection pour les humains et les animaux, et peuvent également contribuer à la contamination de l'environnement par ce parasite. De plus, le risque d'une possible transmission à l'homme est renforcé par l'observation de la contamination des filets.

<u>Ma contribution dans cette étude a été la suivante:</u>

- Réalisation des expériences
- Analyse des données

Identification of *Cryptosporidium* species in fish from Lake Geneva (Lac Léman) in France

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†Died January 2014. This article is dedicated to his memory.

Abstract

Cryptosporidium, a protozoan parasite that can cause severe diarrhea in a wide range of vertebrates including humans, is increasingly recognized as a parasite of a diverse range of wildlife species. However, little data are available regarding the identification of *Cryptosporidium* species and genotypes in wild aquatic environments, and more particularly in edible freshwater fish. To evaluate the prevalence of *Cryptosporidium* spp. in fish from Lake Geneva (Lac Léman) in France, 41 entire fish and 100 fillets (cuts of fish flesh) were collected from fishery suppliers around the lake. Nested PCR using degenerate primers followed by sequence analysis was used. Five fish species were identified as potential hosts of *Cryptosporidium: Salvelinus alpinus, Esox lucius, Coregonus lavaretus, Perca fluviatilis,* and *Rutilus rutilus*. The presence of *Cryptosporidium* spp. was found in 15 out of 41 fish (37%), distributed as follows: 13 (87%) *C. parvum*, 1 (7%) *C. molnari,* and 1 (7%) mixed infection (*C. parvum* and *C. molnari*). *C. molnari* was identified in the stomach, while *C. parvum* was found in the stomach and intestine. *C. molnari* was also detected in 1 out of 100 analyzed fillets. In order to identify *Cryptosporidium* subtypes, sequencing of the highly polymorphic 60-kDa glycoprotein (gp60) was performed. Among the *C. parvum* positive samples, three gp60 subtypes were identified: IIaA15G2R1, IIaA16G2R1, and IIaA17G2R1. Histological examination confirmed the presence of potential developmental stages of *C. parvum* within digestive epithelial cells. These observations suggest that *C. parvum* is infecting fish, rather than being passively carried. Since *C. parvum is a zoonotic* species, fish potentially contaminated by the same subtypes found in terrestrial mammals would be an additional source of infection for humans and animals, and may also contribute to the contamination of the environment with this parasite. Moreover, the risk of human transmission is strengthened by the observation of edible fillet contamination.

Keywords: *Cryptosporidium* spp, wild fish, France

Introduction

Cryptosporidium, a protozoan parasite that can cause severe diarrhea in a wide range of vertebrates including humans, is increasingly recognized as a parasite of a diverse range of wildlife species, including mammals, birds, reptiles, amphibians, and fish [1]. Although the epidemiology of cryptosporidiosis has been widely reported worldwide for different groups of animals, little biological, epidemiological and molecular data are available on *Cryptosporidium* infection in fish, even though the parasite has been already described and

genetically characterized in more than 20 species of both freshwater and marine fish. *Cryptosporidium molnari*, the only currently recognized species infecting fish, was first identified in sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) [2]. *Cryptosporidium scophthalmi* was detected in turbot (*Psetta maxima*, syn. *Scophthalmus maximus*) [3], but this species is still considered a *nomen nudum* due to a lack of genetic data [4].

Cryptosporidium species found in other groups of vertebrates have also been identified in fish, including *C. parvum*, *C. hominis*, *C. scrofarum* and *C. xiaoi*. Additionally, eight *Cryptosporidium* fish genotypes, and one *Cryptosporidium* rat III-like genotype, have been described in fish [4]. Recently, the species name *Cryptosporidium huwi* has been proposed for the piscine genotype 1 from the guppy (*Poecilia reticulata*) to reflect its genetic and biological differences from gastric and intestinal *Cryptosporidium* species [5].

In fish hosts, *Cryptosporidium* fish species and genotypes are located either in the stomach or intestine, as attested by histological analyses. Moreover, it has been reported that the parasite can cause clinical manifestations, such as emaciation, decrease in growth rate, anorexia, whitish feces, abdominal swelling, and ascites [2,3]. An increase in the mortality rate associated with *Cryptosporidium* infection has also been reported, particularly in larval and juvenile infected fish [6]. A significant correlation was found between the presence of the parasite and both fish weight and seasonality, the rate of infection being higher in fish weighing less than 100 grams and in the spring [7]. In addition, a relationship was observed between the presence of the parasite and the production stage in farmed fish [7].

It is notable that many results relating to fish *Cryptosporidium* infection were reported in farmed or aquarium fish [2,7,8]. However, little data are currently available regarding the molecular identification of *Cryptosporidium* species and genotypes in wild fish populations and, in particular, in edible fish. Indeed, only two studies have been conducted in Australia and Papua New Guinea on wild marine and freshwater fish [9,10].

Therefore, the aim of our study was to evaluate the prevalence of *Cryptosporidium* species/ genotypes in freshwater edible fish hosts from Lake Geneva in France. Lake Geneva is located between Switzerland and France, and is the largest freshwater reservoir in Western Europe, with a surface area of 580 km^2, a volume of 89 km^3, and a maximum depth of 309 m (Fig 1). More than 1.5 million people (in France and Switzerland) live around this lake [11]. In addition, the local fish are quite often consumed as raw preparations by the local

population at home or in restaurants located around the shores of the lake. Fish are also a source of income, as around 150 professional fishermen are registered as active on the lake.

Methods

Fish sampling

A total of 41 adult fish were purchased directly on the shores of the lake from local fishermen of Thônon-les-Bains (geographic coordinates: 46° 22' 0" North, 6° 29' 0" East), or Sechex, a small village located eight kilometers West of Thônon-les-Bains, in November 2011 (fall) and April 2013 (spring) (Figure 1). The weight, size, sex, origin and sexual maturity of each individual were determined (Table 1). For each fish, scratchings of the gastric and intestinal epithelia were performed after dissection, and the cells were preserved in the fixative RCL2 and stored at -20 °C. A section of the stomach and bowel were also fixed in 10% buffered formalin. One hundred additional fillets (only cuts of fish flesh without viscera) of European perch (*Perca fluviatilis*) were purchased from the fishermen of Thônon-les-Bains, or St Gingolph (27 km Eeast of Thônon-les-Bains near the Swiss border, geographical coordinates: 46° 23' 0" North, 6° 40' 0" East) to evaluate potential contamination with *Cryptosporidium* spp. at this location. Slices of 2-3 mm were sampled and stored at - 20 °C in RCL2. No approval from Institutional Animal Care and Use Committee or ethics committee was necessary as no experiments that involved alive fish were performed. All fish examined were bought dead from professional fishermen, fishmongers and supermarkets selling fresh fish for consumption. Therefore, no sacrificial method was required. No fish sampled in this work was captured in a protected area and consequently, our sampling protocol did not need any specific permission for the location. Finally, no specimen included in the present work involved endangered or protected species.

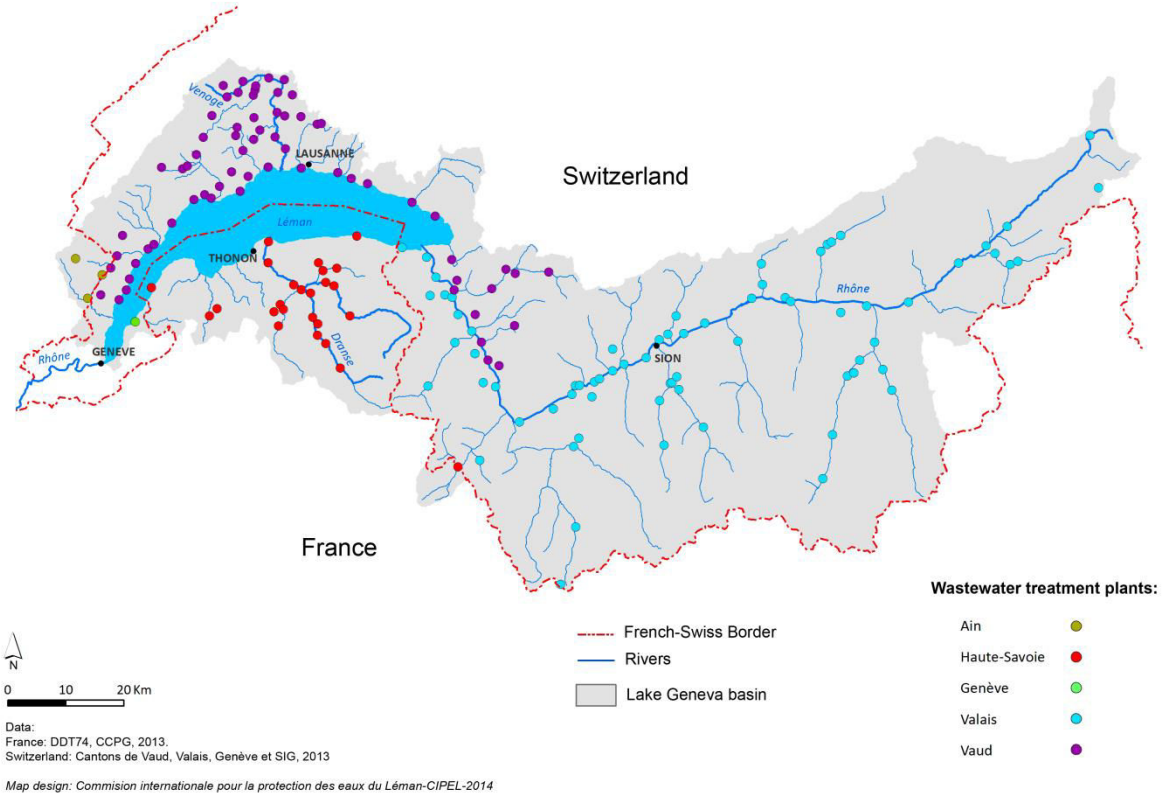

Figure 1. **Map of the study area and sampling site** (Thônon-les-Bains). Effluent from wastewater treatment plants discharged into the Lake Geneva catchment area (CIPEL: Commission Internationale pour la Protection des Eaux du Léman).

Table 2. Freshwater fish specimens collected in Lake Geneva

Fish code	Fish species	Common name	Size (cm)	Weight (g)	Sexual maturity	Sex	Presence of other parasites
5301	*Salvelinus alpinus*	Arctic char	40	610	ND	Male	Cestoda
5302	*Salvelinus alpinus*	Arctic char	37	504	ND	Male	Cestoda
5303	*Salvelinus alpinus*	Arctic char	37	530	ND	Male	Cestoda
5304	*Salvelinus alpinus*	Arctic char	38	548	ND	Male	Cestoda
5305	*Salvelinus alpinus*	Arctic char	29	224	ND	Male	Cestoda
5306	*Salvelinus alpinus*	Arctic char	38	580	ND	Male	Cestoda
5307	*Esox lucius*	Northern pike	37	328	ND	Female	Cestoda
5308	*Esox lucius*	Northern pike	35	328	No	Female	Cestoda
5309	*Lota lota*	Burbot	29	134	ND	Male	Cestoda
5310	*Lota lota*	Burbot	24	96	No	Female	Nematoda
5311	*Coregonus lavaretus*	European whitefish	31	276	No	Female	No
5312	*Coregonus lavaretus*	European whitefish	33	264	ND	Male	No
5313	*Coregonus lavaretus*	European whitefish	29	232	ND	Male	Cestoda
5314	*Coregonus lavaretus*	European whitefish	33	266	ND	Male	Cestoda
5315	*Coregonus lavaretus*	European whitefish	31	220	ND	Male	Cestoda
5316	*Coregonus lavaretus*	European whitefish	22	84	ND	ND	Cestoda
5317	*Perca fluviatilis*	European perch	11	16	ND	Male	Cestoda
5318	*Perca fluviatilis*	European perch	11	18	ND	Male	Cestoda
5319	*Perca fluviatilis*	European perch	11	16	ND	Male	Cestoda
5320	*Perca fluviatilis*	European perch	11	16	ND	ND	Cestoda
5321	*Perca fluviatilis*	European perch	42	1500	Yes	Female	Acantocephala

5322	*Perca fluviatilis*	European perch	29	318	No	Female	Trematoda digenea
5323	*Perca fluviatilis*	European perch	26	220	Yes	Female	Cestoda
5324	*Perca fluviatilis*	European perch	21	124	No	Female	Trematoda digenea
5325	*Rutilus rutilus*	Roach	18	42	No	Female	Cestoda
5326	*Lota lota*	Burbot	31	222	No	Female	No
5327	*Esox lucius*	Northern pike	53	1800	Yes	Female	Cestoda
5328	*Lota lota*	Burbot	25	97	ND	Male	Microsporidia
5329	*Coregonus lavaretus*	European whitefish	40	54	Yes	Female	Cestoda
5330	*Coregonus lavaretus*	European whitefish	33	54	ND	Male	Cestoda
5331	*Coregonus lavaretus*	European whitefish	36	54	ND	Male	Cestoda
5332	*Coregonus lavaretus*	European whitefish	40	54	Yes	Female	No
5333	*Coregonus lavaretus*	European whitefish	39	54	Yes	Female	No
5334	*Esox lucius*	Northern pike	69	2600	ND	Male	Monogenea
5335	*Squalius cephalus*	European chub	52	2300	Yes	Female	Monogenea
5336	*Esox lucius*	Northern pike	60	2000	Yes	Female	Monogenea
5337	*Perca fluviatilis*	European perch	27	30	Yes	Female	Acantocephala
5338	*Abramis brama*	Common bream	50	2000	ND	Male	*Trematoda digenea*
5339	*Perca fluviatilis*	European perch	15	45	Yes	Female	*Trematoda digenea*
5340	*Perca fluviatilis*	European perch	16	47	ND	Male	Cestoda
5341	*Perca fluviatilis*	European perch	16	56	Yes	Female	No

Fish from 5301 to 5326 were purchased from the fishermen of Thônon-les-Bains; Fish from 5327-5341 were purchased from the fishermen of Sechex

ND: Not determined

DNA extraction

Genomic DNA extraction was performed on 96-well plates, using the NucleoSpin™ Kit (Macherey-Nagel, GmbH & Co KG, Germany) according to the manufacturer's protocol. The final DNA elution was 100 µl.

Primer design and nested PCR

An alignment of the 18S rRNA gene sequences obtained from *Cryptosporidium* isolates characterized in fish (GenBank accession numbers: FJ769050, HM243547, HM243548, HM243549, HM243550, JF285332, JF285333, AY524773, HM989832, HM989833, HM989834, HM991857, GQ925452) [7] was performed using the BioEdit v7.0.1 package (http://www.mbio.ncsu.edu:BioEdit/bioedit.html). After identification of a target DNA fragment for nested 18S PCR common to all sequences, two sets of generate primers were selected within the hypervariable region. These degenerate primers were modified from those proposed by Ryan et al [12]. The external primer pair JerExtF (5'-GACATATCWTTYAAGTTTCTGACC-3') (base pair position 292) and JerExtR (5'-CTGAAGGAGTAAGGAACAACC-3') (base pair position 1007) amplified a DNA fragment of 784 bp. The internal primer pair JerIntF (5'-CCTATCAGCTTTMGACGGTAGG-3') (base pair position 289) and JerIntR (5'-TCTAAGAATTTCACCTCTGACTG-3') (base pair position 851) resulted in the amplification of a DNA fragment of 588 bp. For the first round of amplification, the PCR mixture contained 10 µl of DNA, 1x HotStarTaq Plus buffer, 2 mM $MgCl_2$, 0.4 µM for each primer, 200µM dNTP each and 1.5U HotStarTaq® Plus DNA polymerase (Qiagen Inc., Valencia, California) in a final volume of 50 µl. The PCR conditions were as follows: a denaturation step at 94 °C for 10 min, followed by 40 cycles of 94 °C for 45 sec, annealing at 67 °C for 45 sec, and extension at 72 °C for 1 min. The post-extension was completed at 72 °C for 5 min. The second PCR amplification was performed in a 50 µl reaction volume containing 2 µl of the primary PCR product, 1xHotStarTaq Plus buffer, 3 mM $MgCl_2$, 0.4 µM for each primer, 200 µM dNTP each and 1.5 U HotStarTaq® Plus DNA polymerase. The PCR conditions were identical to those in the first round. Nested 18S PCR reactions were conducted using a PTC 200 thermocycler (MJ Research, Waltham, USA). The PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

DNA sequencing and analysis

To identify *Cryptosporidium* species at the molecular level, positive nested 18S PCR products were purified and sequenced directly on both strands, using the forward and reverse primers from the second round, by the company Genoscreen (Institut Pasteur de Lille, France). The sequences obtained were aligned using the BioEdit v7.0.1 package, and then compared with the sequences of *Cryptosporidium* published on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) using the basic local alignment search tool (BLAST) program. Isolates genotyped as *C. parvum* were further subtyped using a second nested PCR that amplifies a fragment of the 60 kDa glycoprotein (gp60) gene, as described [13]. The amplified DNA fragments were purified, sequenced, and analyzed as described above.

Histological analysis

The stomach and intestine of the fish were removed, fixed in 10% buffered formalin, and paraffin-embedded specimens were sectioned to a thickness of 5 µm to be processed using standard staining techniques (Hematoxylin & Eosin). Inflammation in digestive sections was scored as follows: 0, no inflammation; +1, moderate inflammation, focally distributed; +2, moderate inflammation, widely distributed; +3, severe inflammation, widely distributed throughout the section. The sections were examined by a pathologist using a Leica DMRB microscope equipped with a Leica digital camera connected to an Imaging Research MCID analysis system (MCID Software, Cambridge, UK).

Nucleotide sequence accession numbers

The 18S rRNA nucleotide sequences obtained in this study were deposited in the GenBank database under the accession numbers KP939333-KP939354.

Results

The molecular analysis of digestive tissues identified the presence of *Cryptosporidium* spp. in 15 out of 41 fish, representing a frequency of 37%. The fish species Arctic char (*Salvelinus alpinus*) (4/6), Northern pike (*Esox lucius*) (2/5), European whitefish (*Coregonus lavaretus*) (4/11), European perch (*Perca fluviatilis*) (4/12), and roach (*Rutilus rutilus*) (1/1) were identified as potential new hosts for *Cryptosporidium* spp. (Table 2).

Table 2. *Cryptosporidium* species and subtypes in wild freshwater fish from Lake

Code	Fish species	Fish common name	Organ	*Cryptosporidium* species (18S)	Percentage of identity with reference sequences*	SNP position	SNP**	GP60
5302	*Salvelinus alpinus*	Arctic char	Intestine	*C. parvum*	99.8%	347	T/C	NA
5303	*Salvelinus alpinus*	Arctic char	Intestine	*C. parvum*	99.6%	347	T/C	IIaA17G2R1
						435	C/T	
5304	*Salvelinus alpinus*	Arctic char	Stomach	*C. parvum*	99.8%	390	G/A	IIaA15G2R1
			Intestine	*C. parvum*	99.8%	145	A/G	IIaA15G2R1
5305	*Salvelinus alpinus*	Arctic char	Intestine	*C. parvum*	99.6%	300	T/C	IIaA15G2R1
						507	A/G	
5307	*Esox lucius*	Northern pike	Stomach	*C. molnari*	98.3%	314	A/T	NA
						322	T/A	
						324	T/C	
						329	C/T	
						341	A/G	
						370	A/T	
						376	A/T	
						377	C/T	
						506	G/A	
			Intestine	*C. parvum*	99.4%	244	G/A	IIaA17G2R1
						347	T/C	
						496	T/C	
5308	*Esox lucius*	Northern pike	Stomach	*C. molnari*	98.3%	314	A/T	NA
						322	T/A	
						324	T/C	
						329	C/T	
						341	A/G	

						370	A/T	
						376	A/T	
						377	C/T	
						506	G/A	
5311	*Coregonus lavaretus*	European whitefish	Stomach	*C. parvum*	100%	-	-	IIaA15G2R1
			Intestine	*C. parvum*	99.8%	437	T/C	IIaA17G2R1
5312	*Coregonus lavaretus*	European whitefish	Stomach	*C. parvum*	99.6%	324	T/C	IIaA17G2R1
						475	T/C	
	Coregonus lavaretus	European whitefish	Intestine	*C. parvum*	99.2%	87	A/G	-
						151	A/G	
						390	G/A	
						491	T/C	
5314	*Coregonus lavaretus*	European whitefish	Stomach	*C. parvum*	99.8%	235	A/G	-
5316	*Coregonus lavaretus*	European whitefish	Stomach	*C. parvum*	99.8%	390	G/A	-
5318	*Perca fluviatilis*	European perch	Stomach	*C. parvum*	99.6%	300	T/C	IIaA15G2R1
						507	A/G	
5320	*Perca fluviatilis*	European perch	Stomach	*C. parvum*	99.8%	211	C/T	-
5322	*Perca fluviatilis*	European perch	Stomach	*C. parvum*	99.8%	27	G/A	IIaA16G2R1
			Intestine	*C. parvum*	100%	-	-	IIaA16G2R1
5323	*Perca fluviatilis*	European perch	Stomach	*C. parvum*	100%	-	-	IIaA15G2R1
5325	*Rutilus rutilus*	Roach	Stomach	*C. parvum*	100%	-	-	IIaA17G2R1
			Intestine	*C. parvum*	100%	-	-	-

Geneva identified at the 18S rDNA and GP60 loci

*SNP: Single nucleotide polymorphism
** The reference sequences for *C. parvum* and *C. molnari* are: KJ939305 and HM243550 respectively.
NA: Not available

The sequence analysis of the 18S rDNA locus identified two species of *Cryptosporidium*, distributed as follows: 13 *C. parvum* (87%), 1 *C. molnari* (7%), and 1 mixed infection (*C. molnari* and *C. parvum*) (7%). In 9 of the 15 infected fish, the presence of *Cryptosporidium* spp. was found either in the stomach or intestine, while in the 6 remaining infected fish, *Cryptosporidium* spp. were present in both organs. The selective extraction of DNA from these organs, followed by nested 18S PCR and sequencing, confirmed the presence of *C. molnari* only in the stomach of fish, while *C. parvum* was found in both stomach and intestine. Among the stomach samples, two were positive for *C. molnari*, and 10 were positive for *C. parvum*. Among the intestinal samples, eight were positive for *C. parvum* only. The 18S rRNA gene sequences of 5 out of 19 isolates of *C. parvum* found either in the stomach or intestine were 100% identical to that of a previously described species of *C. parvum* (GenBank: KJ939305 [7]), while 14 isolates exhibited single nucleotide polymorphisms (SNPs). It is common to identify sequence differences and variations such as single nucleotide polymorphisms (SNPs) that can be associated to genetic diversity according to the degree of homology. SNPs were distributed as follows: only 1 SNP for 8 isolates, 2 SNPs for 4 isolates, 3 SNPs for 1 isolate, and 4 SNPs for one isolate (Table 2). All SNPs identified in the *C. parvum* isolates corresponded to transition mutations. The two isolates identified as *C. molnari* were identical but showed 9 SNPs in comparison to the *C. molnari* reference sequence (GenBank: HM243550[7] (Table 2). In particular, 5 SNPs were associated with transition mutations, and 4 SNPs (in positions 322, 330, 378, 384) were associated with transversion mutations between adenine and thymine (A/T). The SNPs could not be associated with a specific sampling site (gastric vs. intestinal site) or with a specific fish species.

In order to identify *Cryptosporidium* subtypes, sequencing of the highly polymorphic 60-kDa glycoprotein (gp60) was performed. Partial sequences of the gp60 gene were subsequently obtained for 13 isolates identified as *C. parvum*. Three different subtypes were identified as follows: IIaA15G2R1 (6/13), IIaA17G2R1 (5/13), and IIaA16G2R1 (2/13) (Table 2).

Following histological examination of sections either from the stomach or intestine, the presence of *Cryptosporidium*-like bodies within the cells of the digestive epithelium was confirmed in samples from 10 *C. parvum*-positive fish (Figures 2A, 2B, 2C; Table 3). An inflammatory reaction with leukocyte infiltration was observed occasionally. The presence of other intestinal parasites, identified as nematodes, was confirmed in histological sections of two fish (Figure 2D, Table 2). The histological analysis of the remaining fish was not

possible due to autolysis of tissues.

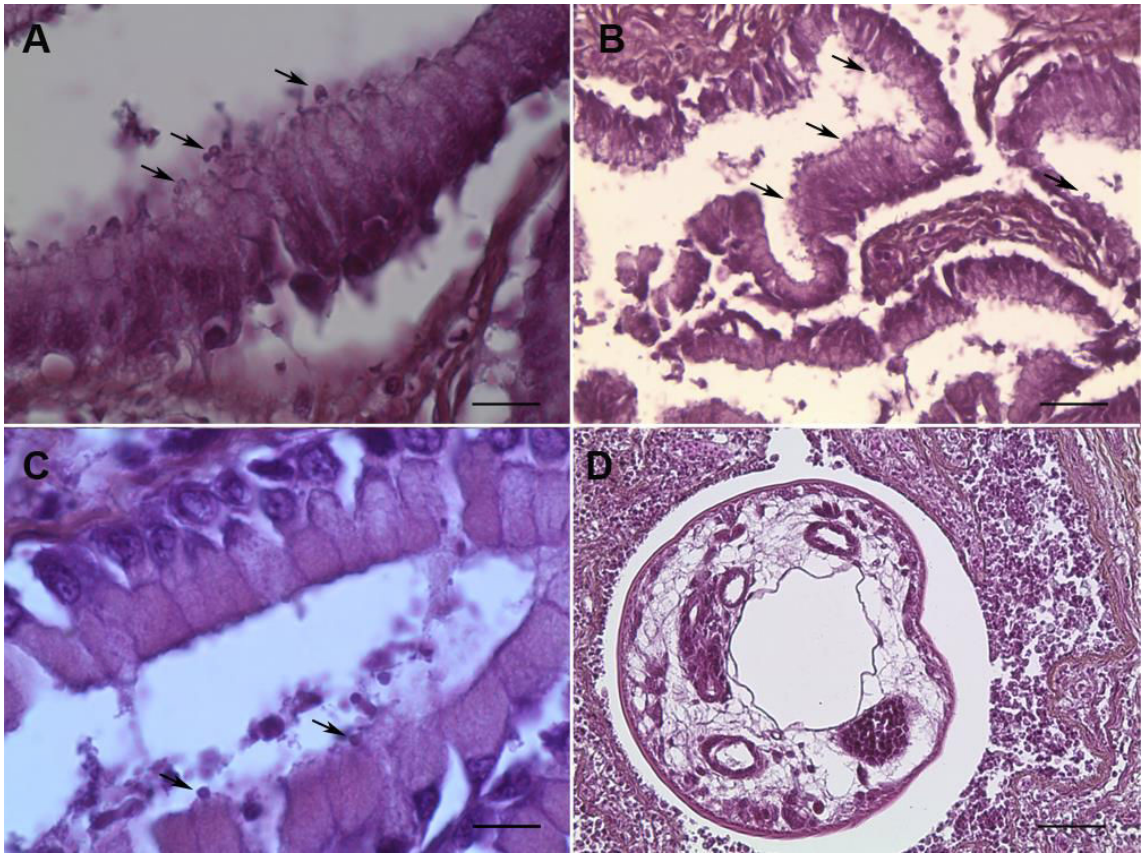

Figure 2. Stained sections of the digestive tract of fish. A. Presence of round bodies suggestive of the developmental stages of *C. parvum* was observed in the apical position (arrows) within the epithelial cells of gastric glands. Bar = 25 µm. B. Presence of round bodies suggestive of the developmental stages of *C. parvum* observed in the apical position (arrows) within the intestinal epithelial cells. Bar = 75 µm. C. Presence of round bodies suggestive of the developmental stages of *C. molnari* observed in the apical position (arrows) within the epithelial cells of gastric glands. Bar = 25 µm. D. Section of a nematode in the intestinal mucosa, surrounded by a severe inflammatory reaction. Bar = 200 µm. Hematoxylin & Eosin staining.

The potential contamination of fish flesh with *Cryptosporidium* spp. was evaluated. One hundred fish fillets of European perch (*Perca fluviatilis*) were analyzed by nested PCR and sequencing. The presence of *C. molnari* was detected in only one fillet. The 18S rRNA gene sequence of this *C. molnari* isolate was identical to that of the two isolates found in the stomach of two Northern pike (Table 2).

Table 3. Histological examination of digestive organs of different fish species from Lake Geneva infected by *Cryptosporidium* spp.

Fish code	Fish species	Fish common name	Organ	Histological examination*	*Cryptosporidium* species (18S rDNA)
5302	*Salvelinus alpinus*	Arctic char	Intestine	Inflammation: +1 Intracellular *Cryptosporidium*-like bodies	*C. parvum*
5303	*Salvelinus alpinus*	Arctic char	Intestine	ND	*C. parvum*
5304	*Salvelinus alpinus*	Arctic char	Stomach	Inflammation: 0 Intracellular *Cryptosporidium*-like bodies	*C. parvum*
			Intestine	ND	*C. parvum*
5305	*Salvelinus alpinus*	Arctic char	Intestine	Inflammation: +1 Intracellular *Cryptosporidium*-like bodies	*C. parvum*
5307	*Esox lucius*	Northern pike	Stomach	ND	*C. molnari*
			Intestine	ND	*C. parvum*
5308	*Esox lucius*	Northern pike	Stomach	Zones of autolysis Inflammation: 0 Intracellular *Cryptosporidium*-like bodies	*C. molnari*
5311	*Coregonus lavaretus*	European whitefish	Stomach	ND	*C. parvum*
			Intestine	Zones of autolysis Inflammation: 0 Intracellular *Cryptosporidium*-like bodies	*C. parvum*
5312	*Coregonus lavaretus*	European whitefish	Stomach	Zones of autolysis Inflammation: 0 Intracellular *Cryptosporidium*-like	*C. parvum*

				bodies	
			Intestine	Zones of autolysis	*C. parvum*
				Inflammation: 0 Intracellular *Cryptosporidium*-like bodies	
5314	*Coregonus lavaretus*	European whitefish	Stomach	Inflammation: 0 Intracellular *Cryptosporidium*-like bodies	*C. parvum*
5316	*Coregonus lavaretus*	European whitefish	Stomach	Inflammation: 0 Intracellular *Cryptosporidium*-like bodies	*C. parvum*
5318	*Perca fluviatilis*	European perch	Stomach	Inflammation : +3	*C. parvum*
				Presence of a nematode	
5320	*Perca fluviatilis*	European perch	Stomach	Autolysis	*C. parvum*
5322	*Perca fluviatilis*	European perch	Stomach	ND	*C. parvum*
			Intestine	Inflammation : +3	*C. parvum*
				Presence of a nematode	
5323	*Perca fluviatilis*	European perch	Stomach	Inflammation: 0 Intracellular *Cryptosporidium*-like bodies	*C. parvum*
5325	*Rutilus rutilus*	Roach	Stomach	Inflammation: 0 Intracellular *Cryptosporidium*-like bodies	*C. parvum*
			Intestine	Inflammation: 0 Intracellular *Cryptosporidium*-like bodies	*C. parvum*

ND: Not done

* Inflammation in digestive sections was scored as follows: 0, no inflammation; +1, moderate inflammation, focally distributed; +2, moderate inflammation, widely distributed; +3, severe inflammation, widely distributed throughout the section.

Discussion

This study reports the first epidemiological and molecular data on the presence of *Cryptosporidium* in fish in France. The overall frequency of *Cryptosporidium* spp. in fish sampled from Lake Geneva was high, reaching 37%. Previous studies have reported a high prevalence of *Cryptosporidium* spp. in fish, but mainly in juvenile marine fish. For instance, Sitjà-Bobadilla et al. reported 100% *C. scophthalmi* prevalence in juvenile turbot in Europe [7]. In contrast, a recent study in Australia found no *Cryptosporidium* isolates in freshwater fish [9], while a *Cryptosporidium* prevalence of 0.2% was found in wild freshwater species in Papua New Guinea [10]. Therefore, even if the comparative data is scarce, this is to our knowledge the first time that *Cryptosporidium* has been detected at a very high prevalence in freshwater fish.

Five new species of fish hosts for *Cryptosporidium* were identified: Arctic char (*Salvelinus alpinus),* Northern pike *(Esox lucius),* European whitefish *(Coregonus lavaretus)*, European perch *(Perca fluviatilis)* and Roach (*Rutilus rutilus).* Although it is generally accepted that the prevalence of *Cryptosporidium* is higher in juvenile fish, all of the fish analyzed in our study were adults, according to size and weight, and according to sexual maturity when this parameter could be determined (Table 1).

Two species of *Cryptosporidium* were detected in fish hosts: *C. molnari* and *C. parvum*. *C. molnari* was identified in freshwater aquaculture fish [14], but this is the first time that this parasite species has been found in wild freshwater fish. The 18S rRNA gene sequences of the 3 *C. molnari* isolates identified in our study were 98% identical to those of the *C. molnari* reference sequences collected from the databases. Interestingly, these three sequences amplified from different individuals presented the same points of mutation, suggesting the circulation of the same parasite isolates in the lake environment.

A matter of importance to public health was the high rate of detection of *C. parvum* among fish hosts, as this species is the most common source of zoonotic infections [4]. Previous studies in Papua New Guinea and Australia also reported consistent detection of *C. parvum* in fish [9,10]. We speculate that the presence of *C. parvum*, and in particular the IIa subtype, in fish samples from Lake Geneva could be due to waterborne contamination with human and animal waste. In fact, the zoonotic *C. parvum* IIa subtype family has predominantly been found in calves and humans in North America, Europe, and Australia [15,16]. In addition, even if we did not search for the presence of *Cryptosporidium* in the lake water, it is well

known that *Cryptosporidium* oocysts are found in groundwater, lakes, rivers, estuaries, and oceans, as a consequence of the great amount of feces from humans, pets, and domesticated or wild animals that is discharged, dumped, or carried in runoff into these waters [17]. In particular, in Lake Geneva, an increase in fecal bacteria of human and animal origin was described in sediment contaminated with wastewater treatment plant effluent, suggesting the presence of both human and animal sources of fecal pollution in the lake environment [11]. In parallel, it has been suggested that when fecal bacteria is present in water, *Cryptosporidium* could be present as well, and even though water quality monitoring and water treatment can reduce the presence of pathogens, they do not ensure absolute safety, due to the fact that *Cryptosporidium* oocysts are highly resistant [18].

Partial sequences of the gp60 gene subsequently amplified from *C. parvum* isolates allowed the identification of three different subtypes belonging to the IIa family, as follows: IIaA15G2R1, IIaA16G2R1, and IIaA17G2R1. The IIaA15G2R1 subtype has also been identified consistently in Papua New Guinea in mackerel scad, *Decapterus maracellus* [10], a wild marine fish. The subtypes IIaA15G2R1 and IIaA17G2R1 have been identified in cattle [19], the first of which is the most dominant zoonotic *Cryptosporidium* subtype infecting dairy cattle and humans in industrialized countries [4]. Indeed, the IIaA15G2R1 subtype represents up to 75% of the identified *Cryptosporidium* subpopulation in French calves [16]. On the other hand, the IIaA16G2R1 subtype has been identified in diarrheic calves [20–22] and also in wild boars (*Sus scrofa*) [23]. In rural areas, it is well known that animals can cohabit with livestock, often by sharing grazing and water sources. Other zoonotic *Cryptosporidium* species already identified in marine fish such as *C. hominis C. xiaoi* and *C. scrofarum* were not found in this study [4].

Since *C. parvum is a zoonotic* species, fish potentially contaminated by the same subtypes infecting terrestrial mammals would be an additional source of infection for humans and other animals, and may also contribute to the contamination of the environment with this parasite. However, it is not clear if fish are only carriers of *C. parvum*, or if *C. parvum* can develop its life cycle and multiply in this fish host.

In order to clarify this question, histological analysis of digestive tissues from *C. parvum*-positive fish was performed, and round bodies suggestive of *C. parvum* developmental stages were observed in an apical position within the cells, either in the stomach or intestine. These observations suggest that *C. parvum* is actually infecting fish, rather than being passively carried. Fluorescent-antibody staining assay using an anti-*Cryptosporidium* antibody (Crypto

Cel immunofluorescence test, Cellabs, Brookvale, New South Wales, Australia) was tried to confirm the detection of *Cryptosporidium* oocysts from fish tissues but unfortunately, no signal was detected. This failure was probably due to the vulnerability of the oocyst antigens to formalin as was already described, particularly after more than one month of formalin fixation which was the case in our study [14]. Mild to moderate inflammation was occasionally found in gastrointestinal tissues, but we could not determine whether it was *Cryptosporidium* that was causing this reaction, since co-infection with other parasites was present. In some cases, the histological analysis of fish was not possible due to autolysis of tissues.

Furthermore, to evaluate a potential contamination of fish fillets with *Cryptosporidium* spp., 100 fish fillets of European perch (*Perca fluviatilis)* were analyzed by nested 18S PCR and sequencing, and the presence of *C. molnari* was detected in fillets from one individual. Fillet contamination with *C. molnari* could occur as a consequence of evisceration of the infected fish during the cleaning and preparation process. Although previous studies have shown no conclusive evidence of transmission of fish-hosted *Cryptosporidium* to mammals [24], the presence of the parasite also in fillets clearly highlights the risk of *Cryptosporidium* infection to humans, either when handling fish or consuming raw or undercooked fish carrying zoonotic species of *Cryptosporidium*. In our study, only *C. molnari*, apparently a non-pathogenic species for humans, was isolated from perch fillets. However, *C. parvum* isolated from the fish digestive tract could certainly be present in fish fillets. Further studies should be done to clarify this aspect.

One study in Maryland consistently reported that urban anglers are at a risk of contracting cryptosporidiosis from exposure received while fishing and consuming caught fish with a mean probability of infection of almost one [25]. Another study showed that blue crabs can transfer *C. parvum* oocysts to people who handle the crustaceans [26]. In addition, it has been reported that immunosuppressed patients are at risk of contracting cryptosporidiosis, either by contact with fish during preparation and handling, or by consumption of undercooked fish [27].

It was not unexpected to find *Cryptosporidium* in fish from Lake Geneva, as this parasite has already been found to be responsible for a human outbreak occurring in 2003 due to the contamination of the water supply network, in the nearby city of Divonne-les-Bains, affecting more than 700 individuals [28]. In addition, in Switzerland, a study reported the presence of

C. parvum in samples collected from the drinking water distribution system in alpine rural regions, and it was suspected that the drinking water was contaminated by grazing cattle [29]. However, future studies should be conducted to detect the presence of the parasite in the lake environment.

In conclusion, these findings suggest that the transmission of *Cryptosporidium* could potentially occur in the interfaces between human, livestock, and fish populations. In fact, the wide host range of *Cryptosporidium* spp., together with the high output of oocyst shedding, allows a high level of contamination of the environment [23]. In particular, for fish hosts, the dispersion and transmission of zoonotic parasites would be facilitated by the aquatic habitat of the host that could potentially release fully sporulated oocysts contributing to the perpetuation of *Cryptosporidium* circulation. Finally, fish may be a good sentinel for the detection of water contamination caused by sewage or agricultural runoff.

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3. Axe 3 : Pathogénicité

1. Article 6 :

Titre: « High Association of *Cryptosporidium* infection with Digestive Cancer in Lebanese patients ».

Préambule : Cet article est en cours de finalisation de rédaction pour soumission..

Résumé :

La cryptosporidiose représente un problème majeur de santé publique. Cette maladie est due à un parasite protozoaire Apicomplexa, *Cryptosporidium*. Outre les diarrhées auto résolutives ou chroniques qu'il entraine respectivement chez les personnes immunocompétentes et immunodéprimées, il a été rapporté qu'une de ses espèces, *Cryptosporidium parvum*, était également capable d'induire des adénocarcinomes digestifs invasifs chez un modèle murin immunodéprimé. A la lumière de cette découverte, nous avons voulu étudier et mettre en évidence chez l'homme, une éventuelle association entre la pathologie cancéreuse et la cryptosporidiose.

Au total, 217 biopsies digestives ont été recueillies auprés de deux laboratoires d'Anatomie et de Cytologie Pathologiques de Tripoli, Liban. Ces biopsies appartiennent à deux groupes de patients: (i) des patients atteints de cancer digestif (estomac ou du côlon) de diagnostic récent et avant tout traitement (n = 92) et (ii) des patients sans cancer digestif, mais avec des symptômes digestifs persistants (n = 125). L'extraction d'ADN a été réalisée à partir de copeaux de biopsies fixées dans le formol et inclus en paraffine, en utilisant le QIAamp DNA Mini kit (Qiagen®) selon les instructions du fabricant, après un traitement au Xylène et une lyse mécanique de l'échantillon. L'ADN de *Cryptosporidium* a été détecté en utilisant une PCR en temps réel ciblant le gène de l'ARNr 18S. Les produits de PCR s'étant révélés positifs au parasite, ont été purifiés puis séquencés pour permettre l'identification des espèces de *Cryptosporidium*. La présence du parasite dans les tissus a été confirmée par observation microscopique de lames histologiques colorées à l'hématoxyline-éosine ou marquées à l'aide d'un anticorps monoclonal anti-*Cryptosporidium* FITC conjugué. Au cours de ce travail les principales observations ont été les suivantes :

1. La prévalence de la cryptosporidiose était de 16.3% (15/92) chez les patients atteints de cancer digestif et 7.2% (9/125) chez les patients sans cancer, mais présentant des troubles gastro-intestinaux. La différence entre les deux groupes a été statistiquement significative (P = 0,047).

2. Après génotypage, deux espèces de *Cryptosporidium* spp ont été identifiées : 19 (79,2%) échantillons correspondant à *C. hominis*, et 5 (20,8%) correspondant à *C. parvum*.

3. La présence du parasite a été mise en évidence dans les zones de lésion néoplasique de 11 des 15 biopsies coliques observées au microscope.

En conclusion, les résultats préliminaires et l'ensemble des données expérimentales chez l'animal et clinico-épidémiologiques chez l'homme montrent que *Cryptosporidium* est étroitement associée au cancer digestif humain. Une forte prévalence de la cryptosporidiose (16.3%) a été détectée chez les patients atteints de cancer digestif et cette prévalence a été encore plus élevée chez les patients atteints de cancer colorectal (21%). La population témoin a montré une prévalence plus faible (6%), similaire à celle de la population générale au Liban. L'étude histologique a permis la confirmation de la présence de différents stades évolutifs du parasite au niveau des lésions néoplasiques. Ces résultats sont cohérents avec ceux rapportées en Pologne décrivant une forte prévalence de la cryptosporidiose chez les patients atteints de cancer colorectal. L'étude de la diversité génétique des isolats a montré une prédominance de *C. hominis* dans les deux populations cible et témoin. Cette distribution est similaire à celle retrouvée dans nos différentes études réalisées au Liban. D'autres études doivent être menées afin de déterminer si le parasite est une cause de la cancérogenèse digestive humaine.

<u>Ma contribution dans cette étude a été la suivante:</u>

- Conception de l'étude
- Réalisation des expériences
- Analyse des données
- Rédaction de l'article

High Association of *Cryptosporidium* infection with Digestive Cancer in Lebanese patients

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Background: Cryptosporidiosis represents a major public health problem caused by the Apicomplexa protozoa, *Cryptosporidium*. This parasite constitutes a significant risk to humans and animals causing self-limited diarrhea in immunocompetent hosts and life threatening disease in immunocompromised hosts. We formerly reported that *C. parvum* strains (of animal and human origin) were able to induce digestive adenocarcinoma in an immunocompromised rodent model even when infections were induced by a single oocyst. With this in mind, we decided to determine the association of *Cryptosporidium* infection with digestive cancer in humans.

Materials and methods: In total, 217 digestive biopsies were collected in two hospitals of Tripoli, Lebanon, from two groups of patients: (i) Patients with digestive cancer (stomach or colon) of recent diagnosis and before any treatment (n= 92) and (ii) Patients without digestive cancer but with persistent digestive symptoms (n= 125). DNA Extraction was performed from Formalin-fixed Paraffin-embedded blocs using the QIAamp DNA Mini Kit (Qiagen®) according to manufacture instructions, after Xylene treatment and a step of mechanic lysis. *Cryptosporidium* DNA was detected in biopsies using a real time PCR. The presence of the parasite in tissue was confirmed by microscopic observation of histological slides stained with Haematoxylin and Eosin and by immunofluorescence analysis using an anti-*Cryptosporidium* monoclonal antibody. To identify *Cryptosporidium* species, positive PCR products from the real time PCR were purified and used to perform multiplex PCR and sequencing.

Results: The prevalence of *Cryptosporidium* infection was 16.3% (15/92) in patients with digestive cancer and 7.2% (9/125) in patients without carcinoma but presenting gastrointestinal disorders. The difference between the two groups was statistically significant (P = 0.047). After genotyping, two *Cryptosporidium* spp. species were found: 19 (79.2%) samples were identified as *C. hominis*, while the remaining 5 (20.8%) were identified as *C. parvum*. After microscopic observation the presence of *Cryptosporidium* was detected in 11 out of 15 samples of colon cancer at the sites of neoplastic lesions.

Conclusion: These preliminary results show that *Cryptosporidium* is strongly associated with human digestive cancer. Further studies should be done to determine if the parasite is a cause of human digestive carcinogenesis.

Introduction:

According to the World Health Organization (WHO), cancer is the leading cause of death worldwide. In 2012, more than 14.1 million new cases and 8.2 million deaths due to cancer (around 13% of all deaths) were reported. In developing countries this rate is particularly high and cancer can lead to death in more than 70% of cases (WHO, 2014). Many interacting factors that cause cancer and are able to induce the transformation of a normal cell include genetic factors; lifestyle factors such as tobacco use, diet, and physical activity; environmental exposures to different types of chemicals and radiation and certain types of infections (Burt and Neklason, 2005). Particularly, WHO acknowledges that 20% of cancers are due to infectious agents (Banuls et al., 2013). Around 2 million of human cancer cases in 2008 were due to viral, bacterial and parasitic infections (Benamrouz et al., 2012a).

Helicobacter pylori, Hepatitis B and C viruses, Human Papilloma viruses and Epstein-Barr virus are responsible of the majority of microbial induced-cancer and have been identified as carcinogenic to humans in the International Agency for Research on Cancer (IARC) (De Martel et al., 2012; WHO, 2014). However, parasites including helminthes are al so recognized as oncogenic. Among them, *Schistosoma haematobium* has been causally associated with urinary bladder cancer, and the flukes *Opisthorchis viverrini* and *Clonorchis sinensis* were causally associated to cholangiocarcinoma in human population (De Martel et al., 2012). Based on clinical and epidemiological evidences, many reports underlined a potential association between parasitic protozoan infections and cancer. Thus, it is suggested that *Plasmodium* could play a role in the development of Burkitt lymphoma (Bouvard et al., 2012). In addition, *Theileria annulata* and *Theileria parva*, apicomplexan parasites, were clearly shown to be able to induce a host cell malignant transformation leading to uncontrolled proliferation and allowing clonal expansion of leucocytes and epithelial cells in animals (Dobbelaere and Rottenberg, 2003).

Our team has discovered that *Cryptosporidium parvum*, a species frequently isolated from human and animal digestive samples, is able to induce digestive adenocarcinoma in SCID mice (Certad et al., 2010a; Certad et al., 2010b; Certad et al., 2007). Cryptosporidiosis represents a major public health problem caused by the Apicomplexa protozoa, *Cryptosporidium*. This parasite constitutes a significant risk to humans and animals causing self-limited diarrhea in immunocompetent hosts and life threatening disease in immunocompromised hosts. For the first time, it was demonstrated that an eukaryotic

microorganism was involved in neoplastic changes in the digestive epithelium of a mammalian host. Herein, adenomas with low or high grade intraepithelial neoplasia, intramucosal or invasive adenocarcinoma, always associated with *C. parvum* parasites, were detected in the digestive tract of SCID mice, including stomach and ileocaecal region, even when infection was induced with one oocyst (Benamrouz et al., 2012b). Consistently, epidemiological studies were conducted in Poland and a high frequency (12%) of cryptosporidiosis in patients with colorectal cancer was reported (Sulzyc-Bielicka et al., 2012). However, the correlation between cryptosporidiosis and human digestive cancer remains unclear and it is not evident if this intracellular parasite considered as an opportunistic agent is able to induce gastro-intestinal malignancies in humans or if its presence is a consequence of an immune deficiency due to cancer. Interestingly, in the same experimental model, the species C. *muris* with gastric tropism (infecting mice and humans) could not induce that type of epithelial cell transformation (Certad et al., 2007). However, when *C. hominis* was tested in this model, infections did not develop although a good oocyst viability was observed (>80%) before inoculation (Certad et al., 2010a).

In fact, experimental observations in the rodent model, and some reports that suggest an association of cryptosporidiosis with cancer in humans, largely justify clinical research aiming at exploring the causal involvement of *Cryptosporidium* spp in colorectal cancer (CRC).

In order to add new strong arguments for a probable association between cryptosporidiosis and digestive human cancer, the main aim of this study was to determine prevalence and to identify species of *Cryptosporidium* among a Lebanese digestive cancer population.

Material and Methods:

Ethics:

Authorization to conduct this study was obtained from the Lebanese Minister of Public Health (reference number: 4-39716). The institutional directory review boards of Pathology laboratories in Tripoli also approved the protocol of this project in agreement with Lebanese legislation. Written informed consents were obtained from all patients after a clear explanation of the research objectives. The present study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Subjects and samples collection

In total, 217 digestive biopsies embedded in paraffin were collected in two pathology laboratories of Tripoli, Lebanon, from two groups of patients: (i) Patients with digestive cancer of recent diagnosis and before any treatment (n= 92: 71 colon biopsies and 21 gastric biopsies) and (ii) Patients without digestive cancer but with persistent digestive symptoms (n= 125: 82 colon biopsies and 43 gastric biopsies). All patients were HIV (Human Immunodeficiency Virus) negative.

Histological analysis

Sections of 5 µm thick from biopsies embedded in paraffin were stained by haematoxylin-eosin. Sections were examined and the presence of parasites and histological lesions was verified. When neoplastic lesions were present at different sites these lesions were scored according to the ‘‘Vienna classification’’ of the epithelial neoplasia of the gastrointestinal tract , as previously described (Scoazec, 2007). A Leica DMRB microscope equipped with a Leica digital camera connected to an Imaging Research MCID analysis system (MCID software, Cambridge, United Kingdom) allowed the obtaining of photomicrographies.

DNA extraction from formalin-fixed paraffin-embedded tissue samples.

DNA was extracted from a mixture of 2 sections of 30µm from each block. Histologic sections were processed by using xylene and ethanol for paraffin removal and were then rehydrated. To disrupt the wall of potential oocysts, the samples were lysed during 70 seconds using glass beads, acid-washed 425-600 µm (Sigma Aldrich, Germany). DNA was then extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer’s recommended procedures with slight modifications: the proteinase K digestion was performed overnight and in addition a formalin disassembly step at 90°C for 1 hour was added. The DNA was eluted in 100 µl of elution buffer (Qiagen) and stored at 4°C until use.

***Cryptosporidium* molecular detection and species identification**

All samples were used for molecular detection of *Cryptosporidium.* The 18S rRNA real-time PCR was performed with primers and TaqMan probe, as previously described by Mary *et al* (Mary et al., 2013). The potential presence of PCR inhibitors in specimens was tested.

To identify *Cryptosporidium* spp. molecularly, positive PCR products were purified and sequenced directly by the company Genoscreen (Pasteur Institute, Lille) on both strands using the forward and reverse primers used for the PCR. The sequences obtained were aligned using the BioEdit v7.0.1 package (http://www.mbio.ncsu.edu:BioEdit/bioedit.html), then compared with sequences of *Cryptosporidium* published on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) using the basic local alignment search tool (BLAST) program.

Direct Immunofluorescence detection of *Cryptosporidium* spp oocysts

Histological sections of 5 µm thick from biopsies embedded in paraffin were tested for the detection of *Cryptosporidium* oocysts using a fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* sp. MAbs (Cellabs, Brookvale, New South Wales, Australia) according to the manufacturers' instructions. Slides were visualized by using a Zeiss LSM780 confocal microscope equipped for FITC fluorescence (maximum excitation wavelength, 490 nm)

Real time PCR for Epstein barr virus (EBV) detection

Genomic detection and quantification of the EBV genome was performed from 10µl of DNA using the quantitative EBV R-gene test system (Argene, bioMérieux Marcy l'Etoile, France) according to the manufacturers' instructions.

Statistical analysis

Categorical data were presented as proportions. Categorical variables were compared using Fisher's exact test. Logistic regression model was performed to calculate odds ratios with *Cryptosporidium* infections as the main outcome. The general significance level was set at P-value below 0.05.

Results:

A total of 217 biopsies were collected in this study: 92 (71 from colon and 21 from stomach) from patients with a newly diagnosed digestive cancer and without treatment and 125 (82 from colon and 43 from stomach) from subjects without digestive cancer but with persistent digestive symptoms. The age of patients was between 18 and 92 years old (mean of age: 50 ± 19). No significant differences related to age or sex were observed between the groups. Table 1 demonstrates the demographic characteristics of the studied patients.

Table 1. Demographic characteristic of the population

	Biopsies from patients with colon or stomach neoplasia or cancer	**Biopsies from patients with other digestive pathology (without cancer)**
N		
Patients with colorectal samples	71	82
Patients with stomach samples	21	43
Total patients	92	125
Age		
Patients with colorectal samples	59.8 ± 15.8 years	49.6 ± 18.5 years
Patients with stomach samples	70.8 ± 15.6 years	39.4 ± 16 years
Total patients	62.3 ± 15.6 years	46.1 ± 18.4 years
Sex		
Male (%)	37 (40.2%)	56 (44.8%)
Female (%)	55 (59.8%)	69 (55.2%)

After using qPCR, the prevalence of *Cryptosporidium* infection was detected in 16.3% (15/92) of patients with digestive cancer and in 7.2% (9/125) of patients without carcinoma but presenting persistent gastrointestinal disorders. The difference between these two groups was statistically significant ($P = 0.047$).

When localization of cancer was considered, *Cryptosporidium* infection was found in colonic biopsies from 21.1% (15/71) of patients with cancer compared to 6.1% (5/82) from the control group being this difference statistically significant ($P = 0.008$). Concerning gastric biopsies, *Cryptosporidium* infection was no detected but the presence of the parasite was found among samples classified as gastritis. The results are summarized in Tables 2 and 3.

After genotyping, two *Cryptosporidium* spp. species were found: 19 (79.2%) samples were identified as *C. hominis*, while the remaining 5 (20.8%) were identified as *C. parvum*. The sequences obtained showed 100% of identity with the reference sequences. Regarding colon biopsies, 12 (80%) and 4 (80%) were identified as *C. hominis*, while the remaining 3 (20%) and 1 (20%) were identified as *C. parvum* in carcinoma and non-carcinoma populations, respectively. Similarly, among gastric biopsies population, we identified 3 isolates as *C. hominis* (75%) and only one as *C. parvum* (25%). *Cryptosporidium* spp. other than *C. parvum* and *C. hominis* were not found. No significant differences related to species were observed.

The presence of the parasite in tissues was confirmed by microscopic observation of histological slides of colonic sections stained with Haematoxylin and Eosin in 11 out 15 cases of colon cancer at the sites of neoplastic lesions and by immunofluorescence analysis using an anti-*Cryptosporidium* monoclonal antibody (Figure 1).

As a comparison, biopsies from 8 cancerous patients and 2 non-cancerous control subjects were also analyzed for detection of EBV DNA by real-time PCR. For technical reasons only results from 10 samples were obtained. Results are summarized in Table 4.

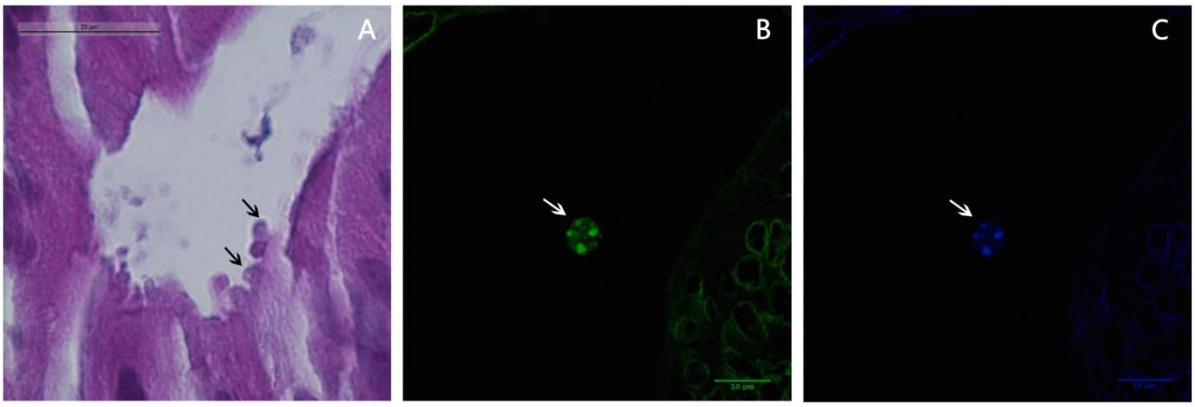

Figure 1: Histological sections of the colon cancer region in biopsies specimens from patients with digestive cancer and *Cryptosporidium* infection according to molecular analysis (A) Several developmental stages of *C. parvum* were observed in the apical position (arrows) within the epithelial cells of intestinal glands. (Haematoxylin and Eosin straining, Bar = 20 µm). (B) Presence of a *Cryptosporidium* oocyst is shown (arrow) in the lumen of an intestinal gland after immunofluorescence using a fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* spp. MAbs. (Bar = 10 µm). (C) Four sporozoites in the oocyst with presence of one nucleus in each sporozoite were observed (arrow) after staining with 4,6-diamidino 2-phenyl-indole dihydrochloride (DAPI). (Bar = 10 µm).

Table 2. *Cryptosporidium* prevalence in biopsies from patients with digestive cancer compared to biopsies from patients with other digestive pathology

	N (%) Biopsies from patients with colon or stomach neoplasia or cancer	**N (%) Biopsies from patients with other digestive pathology (without cancer)**	**Fisher's exact (*P-value*)**
***Cryptosporidium* negative**	77 (84%)	116 (93%)	**0.047**
***Cryptosporidium* positive**	15 (16%)	9 (7%)	
Total	92	125	

Table 3. *Cryptosporidium* prevalence in biopsies from patients with colonic cancer compared to patients without colonic cancer

	N (%) Biopsies from patients with colorectal neoplasia or cancer	N (%) colorectal biopsies from patients with other digestive pathology (without cancer)	Fisher's exact (*P-value*)
***Cryptosporidium* negative**	56 (79%)	77 (94%)	**0.008**
***Cryptosporidium* positive**	15 (21%)	5 (6%)	
Total	71	82	

Table 4. *Cryptosporidium* and EBV co-infection in biopsies from patients with cancer compared to patients without colonic

	N Biopsies from patients with neoplasia or cancer		N Biopsies from patients with other digestive pathology (without cancer)	
	EBV negative	**EBV positive**	**EBV negative**	**EBV positive**
***Cryptosporidium* negative**	1	2	0	0
***Cryptosporidium* positive**	2	3	1	1

Discussion:

Cryptosporidium has been described as the second biggest cause of diarrheal disease and death in infants under 1 year after Rotavirus (Striepen, 2013). In addition, an increase in the incidence of cryptosporidiosis in general population has been reported recently in U.S.A, mainly due to a dramatic rise in community outbreaks (Yoder & Beach, 2010).

However our study reports new epidemiological evidence for a link between *Cryptosporidium* infection and digestive cancer. The data herein described a high rate (21%) of cryptosporidiosis in Lebanese patients with colorectal cancer being this prevalence higher when compared with the burden of cryptosporidiosis in the control group (6%). However, in this study the parasite was not detected in gastric biopsies from subjects with cancer.

Interestingly, this prevalence of *Cryptosporidium* detected in these patients is also higher when compared with prevalence reported in previous Lebanese epidemiological studies: 10% in symptomatic patients and in schoolchildren and 5% detected in the general population (Osman et al., 2015) (Osman et al, Schoolchildren/Transmission), while the prevalence in the control group of symptomatic patients is in the range of that of the general population (Osman et al., 2015).

In order to exclude other oncogenic pathogens maybe responsible for colon cancer such as Epstein barr virus (EBV) (Fiorina et al., 2014), real time PCR detection was performed. Due to technical reasons only results from few samples were obtained but these preliminary results showed an homogenous distribution of the virus among groups of *Cryptosporidium* infected and non-infected patients, as well as among the subjects with and without cancer, consistent with the fact that the virus infects almost everyone in early life persisting throughout life (Parkin, 2011). Further studies should be done to clarify this aspect.

On the other hand, the molecular characterization of all *Cryptosporidium* isolates allowed the identification of two species: *C. hominis* and *C. parvum*, with the former being predominant (79.2%). These results confirm recent reports in Lebanon showing the presence of these two species with predomination of *C. hominis* (Osman et al., 2015). However, for the first time *C. hominis* is associated with colon cancer. However the identification of subtypes could not be possible because the DNA extracted from biopsies is fragmented and the realizing of the PCR targeting the gp60 gene was not possible.

Until now the relation between colorectal cancer and cryptosporidiosis has not been well established. Because *Cryptosporidium* is an opportunistic agent that causes significant morbidity and mortality in immunocompromised patients, it is possible that individuals infected with this parasite may have a higher risk of developing malignancies, especially when immunosuppression is more severe.

There are some speculations in the literature about possible associations between *Cryptosporidium* infection and digestive neoplasia in different populations: The association of cryptosporidiosis and colonic adenocarcinoma was suspected in the case of a Spanish patient carrying both, who died rapidly after the onset of symptoms (Izquierdo et al., 1988). A cryptosporidiosis case of the biliary tract clinically mimicking a pancreatic cancer in an AIDS patient was also described (de Souza Ldo et al., 2004).

In addition, in a retrospective study among HIV-infected subjects known to be highly susceptible to *Cryptosporidium* infections, the incidence of colorectal cancer was found to be 2.3 fold (1.8–2.9) higher than in the general population. These patients developed tumors at earlier ages in comparison to immunocompetent persons. Unfortunately, no data about gastro-intestinal parasites in these patients were available (Patel et al., 2008). A recent study reported that the risk of developing a colon carcinoma is significantly elevated among AIDS patients presenting cryptosporidiosis (Shebl et al., 2012). A possible association between human cryptosporidiosis and liver cancer (bile duct carcinoma) was suggested in children with X-linked hyper-IgM syndrome. These authors proposed that the mutation responsible for this defect has a role in the colonization of the biliary epithelium by different pathogens, including *Cryptosporidium*. A chronic infection and inflammation by this parasite will follow, perhaps being the inflammation the cause of the oncogenicity (Hayward et al., 1997).

Furthermore, epidemiological studies in Poland reported a frequency of 18% and 12.6% of cryptosporidiosis in patients with colorectal cancer of recent diagnosis before any immunosuppressive treatment. In these studies the species responsible for infection was not determined (Sulzyc-Bielicka et al., 2012; Sulzyc-Bielicka et al., 2007).

Moreover, previous works have associated cryptosporidiosis with the development of intestinal polyps in naturally infected sheep (Gregory et al., 1987) and in aural-pharyngeal pedunculated polyps of iguanas but without signs of malignancies (Uhl et al., 2001). The occurrence of dysplasia in the biliary tree epithelia was described in an experimental model of IFN-δ knockout mice infected with *Cryptosporidium* (Stephens et al., 1999). The authors

proposed that the response of these mice to *Cryptosporidium* infection may model the initial steps towards the development of cholangitis and bile duct related cancer in patients with immunodeficiency (Stephens et al., 1999). More recently, other investigators also reported the association between *Cryptosporidium* and digestive cancer in a model of Dexamethasone treated immunocompetent Swiss albino mice (Abdou et al., 2013)**.** Moreover, the ability of *C. parvum* to induce gastrointestinal neoplastic changes was established experimentally in a SCID model as explained above (Benamrouz et al., 2012b; Certad et al., 2012; Certad et al., 2010a; Certad et al., 2010b; Certad et al., 2007).

In fact, the potential role of *Cryptosporidium* in the development of neoplasia is not surprising considering that several studies have reported about the capacity of some protozoa to interfere with signaling pathways of the host cell. To favor their survival and transmission during the long years they must spend in their host (Banuls et al., 2013). For instance, *C. parvum* was shown to be able of activating NF-κB pathway in directly infected cells, preventing the induction of apoptosis after infection using an in vitro model of biliary cryptosporidiosis. NF-κB family of transcription factors regulates the activation of a number of intracellular survival signals including the c-Myc proto-oncogene. Indeed, activation of NF-κB has been observed in many types of cancer, including colon cancer. However, it has been found in in-vitro studies that *C. parvum*, depending on its developmental stage, not only inhibits but it can modulate host-cell apoptosis, inhibiting apoptosis at the trophozoite stage and promoting this process at the sporozoite and merozoite stages. Modulation of apoptotic pathways was also investigated by microarray analysis in in-vitro model using human ileocaecal HCT8 cells. Genome wide expression profiling revealed high proportion of apoptosis genes regulated during *C. parvum* infection. Indeed, resistance to apoptosis could be an essential step in the progression to malignancy (Benamrouz et al., 2012a).

Interestingly, it is well known that the parasite induces modifications of the host actin cytoskeleton of intestinal epithelial cells although little information is available about the significance of the host actin remodeling process. In consistence, recent data found hat *C. parvum*, independently of the strain, is able to modulate host cytoskeleton activities and several host-cell biological processes via the Wnt signaling pathway in SCID mice (Benamrouz et al., 2014).

In conclusion, we provide the first molecular data showing a high association between cryptosporidiosis and digestive cancer, especially colorectal cancer. *C. hominis* was reported

for the first time as associated with colon cancer. Research on this topic could be worthwhile since the incidence of *Cryptosporidium* infection seems to increase not only amongst immunosuppressed patients but also in the general population worldwide.

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2. Article 7 :

Titre: « *Cryptosporidium parvum*-induced ileo-caecal adenocarcinoma and WNT signaling in a rodent model ».

Préambule : Cette étude a fait l'objet d'une publication dans le journal Diseases Models and Mechanisms. 2014 ; 7(6), 693-700.

Résumé :

Cryptosporidium est un protiste cosmopolite très répandu dans le monde. Les espèces de ce parasite constituent un risque d'infection important pour l'homme et les animaux. De récentes données ont permis de décrire un pouvoir carcinogène de différentes souches (d'origine animale et humaine) de l'espèce *C. parvum* chez un modèle murin immunodéprimé, même avec de très faibles quantités de parasite (un seul oocyste). Le but de cette étude était de caractériser les lésions néoplasiques induites par l'infection par *C. parvum* et d'explorer les voies métaboliques potentiellement impliqués.

Pour ce faire, nous avons suivi la progression des lésions chez les souris SCID-D infectées avec différentes souches de *C. parvum*. De plus, nous avons recherché par analyse immunohistochimique, microscopie électronique et par biologie moléculaire, la présence d'altérations dans des gènes ou dans l'expression de protéines couramment impliqués dans le cycle, la différenciation ou la migration cellulaire, tels que la Bêta-caténine, l'APC, K-ras et la p53.

1. L'analyse immunohistochimique a pu montrer une localisation anormale des composants de la voie de signalisation WNT et p53 au fur et mesure de la progression et de la sévérité de la lésion néoplasique.

2. Par contre, aucune mutation au niveau des *loci* décrits comme étant fréquemment mutés dans les cas de cancer colorectaux, n'a pu être mise en évidence.

3. De plus, l'analyse en microscopie électronique à transmission a permis de montrer des altérations ultra-structurales au niveau des jonctions d'adhésion de l'épithélium néoplasique iléo-caecale de souris infectées par *C. parvum*.

En conclusion, dans cette étude, nous avons mis en évidence pour la première fois que la voie de signalisation Wnt, ainsi que le cytosquelette de la cellule hôte étaient impliqués dans le développement du processus néoplasique induit par *C. parvum* et la migration cellulaire des cellules transformées.

Ma contribution dans cette étude a été la suivante:

- Réalisation des expériences
- Analyse des données

Cryptosporidium parvum-induced ileo-caecal adenocarcinoma and WNT signaling in a rodent model

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SUMMARY

Cryptosporidium species are worldwide spread apicomplexan protozoan. These parasites constitute a significant risk to humans and animals. They cause self-limited diarrhea in immunocompetent hosts and a life threatening disease in immunocompromised hosts. Interestingly, *Cryptosporidium parvum* has been related to digestive carcinogenesis in humans. Consistently with a potential tumorigenic role of this parasite, in an original reproducible animal model of chronic cryptosporidiosis based on dexamethasone-treated or untreated adult SCID mice, we formerly reported that *C. parvum* (strains of animal and human origin) is able to induce digestive adenocarcinoma even in infections induced with very low inoculum. The aim of this study was to further characterize this animal model and to explore metabolic pathways potentially involved in the development of *C. parvum*-induced ileo-caecal oncogenesis. We searched for alterations in genes or proteins commonly involved in cell cycle, differentiation or cell migration, such as β-catenin, *Apc*, E-cadherin, *Kras* and p53. After infection of animals with *C. parvum* we demonstrated immunohistochemical abnormal localization of Wnt signaling pathway components and p53. Mutations in the selected *loci* of studied genes were not found after high-throughput sequencing. Furthermore, alterations in the ultrastructure of adherens junctions of the ileo-caecal neoplastic epithelia of *C. parvum* infected mice were recorded using transmission electron microscopy. In conclusion, we found for the first time that the Wnt signaling pathway, and particularly the cytoskeleton network seems to be pivotal for the development of *C. parvum*-induced neoplastic process and cell migration of transformed cells. Furthermore, this model is a valuable tool to contribute to the comprehension of the host-pathogen interactions associated to the intricate infection process due to this parasite, which is able to modulate host cytoskeleton activities and several host-cell biological processes and that remains a significant cause of infection worldwide.

INTRODUCTION

Cryptosporidium species are worldwide spread apicomplexan parasitic protists. The infection results from the ingestion of *Cryptosporidium* oocysts through the consumption of fecally contaminated food or water or through direct person-to-person or animal-to-person contact (Chalmers and Katzer, 2013). This ubiquitous, intracellular parasite constitutes a significant health risk to humans and animals. It causes self-limited diarrhea in immunocompetent persons and a life threatening disease in immunocompromised persons (Ramirez et al., 2004). Contaminated water is the major source of *Cryptosporidium* infections for humans. Large-scale outbreaks of human cryptosporidiosis were reported, often implicating contaminated drinking or recreational water (Ramirez et al., 2004,Rowan, 2011,Yoder and Beach, 2010). The ingestion of as few as ten oocysts can cause infection in immunocompetent persons (Okhuysen et al., 1999). This low infection threshold, together with the well-known resistance of *Cryptosporidium* oocysts to chlorine disinfection at concentrations typically applied in drinking water plants, facilitate the waterborne transmission of cryptosporidiosis (Rowan, 2011,Yoder and Beach, 2010).

Nevertheless, key aspects of cryptosporidiosis remain unclear. For this reason, to contribute to the understanding of the dynamics of the infection, we formerly developed an animal model of cryptosporidiosis using dexamethasone-treated or untreated adult SCID mice orally infected with *Cryptosporidium parvum* or *C. muris* oocysts. Unexpectedly, we observed that *C. parvum*-infected SCID mice developed digestive adenocarcinoma (Certad et al., 2007). Low or high grade intraepithelial neoplasia and invasive adenocarcinoma associated with numerous *C. parvum* life stages were detected in the digestive tract of SCID mice, including stomach, ileo-caecal region and intrahepatic biliary tree (Certad et al., 2012,Certad et al., 2010a,Certad et al., 2010b,Certad et al., 2007). A highly significant correlation was found between the extension of cryptosporidiosis and the severity of neoplastic lesions (Certad et al., 2010b).

Further analysis allow us to show that different strains of *C. parvum* isolated from either animals or humans, induced digestive neoplasia in this rodent model (Certad et al., 2012,Certad et al., 2010a,Certad et al., 2010b,Certad et al., 2007), even in infections induced with very low inoculum sizes (1 – 10 oocysts) (Benamrouz et al., 2012). On the other hand, in the same experimental model, the species *C. muris* with gastric tropism (infecting mice

and humans) did not induce that type of epithelial cell transformation independently of the grade of immunosupression (Certad et al., 2010b).

Consistently, different evidences have shown direct or indirect association between cryptosporidiosis and cancer in different human populations: In a study among HIV-infected subjects known to be highly susceptible to *Cryptosporidium* infections, the incidence of colorectal cancer was found to be higher than in the general population (Patel et al., 2008). Another study reported that the risk of developing a colon carcinoma is significantly elevated among AIDS patients presenting cryptosporidiosis (Shebl et al., 2012). A possible association between human cryptosporidiosis and liver cancer was suggested in children with X-linked hyper-IgM syndrome (Tomizawa et al., 2004). Two epidemiological studies in Poland reported a frequency of 18% and 12.6% of cryptosporidiosis in patients with colorectal cancer of recent diagnosis before any immunosuppressive treatment (Sulżyc-Bielicka et al., 2012,Sulzyc-Bielicka et al., 2007).

As well, it has been shown experimentally that *C. parvum* infection alters gene profile expression of the host cell. These altered genes include those associated to apoptosis such as BCL2 and the c-Myc proto-oncogene (Liu et al., 2009), proinflammatory signaling cascades and cytoskeletal dynamics (Deng et al., 2004). Nevertheless, even if we can hypothesize that the acquired transformed phenotype of *Cryptosporidium* infected epithelial cells is a consequence of modulation of cell signaling by the parasite, to our knowledge, no data about the mechanism of *C. parvum* induced neoplasia are available.

The present work belongs to a series of experiments exploring the ability of *C. parvum* to induce neoplastic changes in the digestive epithelium of the animal model. The aim of this study was to further characterize this animal model and to explore metabolic pathways potentially involved in the development of ileo-caecal neoplasia induced by *C. parvum* infection, which is to our knowledge the first parasitic protiste able to induce epithelial invasive neoplasia in mammals (Benamrouz et al., 2012,Certad et al., 2012,Certad et al., 2010a,Certad et al., 2010b,Certad et al., 2007). We searched for alterations in genes or proteins commonly involved in cell cycle, differentiation or cell migration, such as β-catenin and *Apc* (components of the Wnt signaling pathway), the *Kras* oncogene and p53 by molecular and immunohistochemical approaches.

RESULTS

Development of intraepithelial neoplasia and adenocarcinoma

The histopathological study showed the development of neoplasia of different grades of severity. Observed lesions varied from LGIEN to well differentiated invasive adenocarcinoma. A total of 27 Dex-treated SCID mice were successfully infected with different strains of *C. parvum* (IOWA: 20 mice, TUM1: 4 mice and IIaA15G2R1 human isolate: 3 mice).

At histological examination of the ileo-caecal region we observed LGIEN in 2/19 mice. The detected lesions were characterized by slight modified mucosal architecture, including irregular glands lined by cells with slight atypias and containing minimal mucin or depletion. In 8/19 animals, we discovered exophytic adenomas showing an increasing architectural distortion, glandular crowding and major cellular atypias (HGIEN). In 8/19 mice, adenocarcinoma processes invading the submucosae though the muscularis mucosae were observed. In 1/19 mouse we observed a well differentiated adenocarcinoma invading the inner layer of the muscularis. In general, lesions showed a gradual progression from LGIEN to HGIEN and invasive well-differentiated adenocarcinoma progressing into the lamina propria (intramucosal carcinoma), into the submucosa and through the muscularis-mucosae into the subserosa. The severity of lesions increased steadily according to the delay P.I.. Those neoplastic lesions were accompanied by a diffuse inflammatory cell infiltrate, particularly in mice infected with TUM1 and II2A15G2R1 strains. In summary, the incidence of ileo- caecal neoplasia was present in 19/19 (100%) infected animals euthanatized after 40 days P. I.

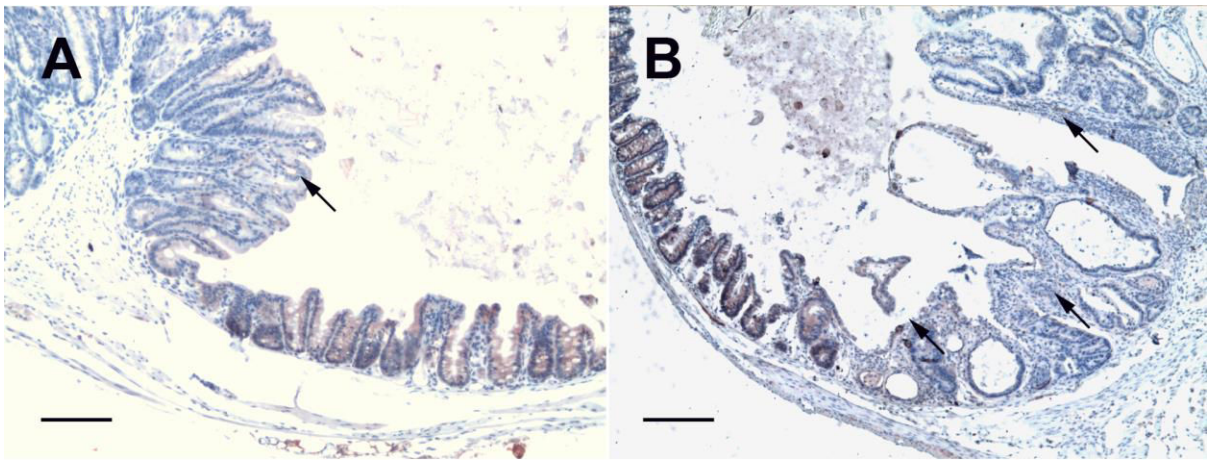


Fig. 1. Section of ileo-caecal regions of Dex-treated SCID mice immunostained for *Apc*. (A) Polypoid-adenoma section from a mouse euthanatized at 45 days P. I. showing a decrease of the intensity of cytoplasmic *Apc* labeling (arrow) after infection with *C. parvum*, while contiguous normal mouse tissue showed a staining pattern similar to that seen in a normal mucosa. Bar= 100 µm. (B) In a section of the ileo-caecal region of a *C. parvum* infected mouse at 60 days P. I. polypoid-adenoma section showing a decrease of the intensity of cytoplasmic *Apc* labeling (arrow). Bar= 100 µm.

Apc labeling

In the mucosa of ileo-caecal regions of uninfected control mice, *Apc* cytoplasmic immunoreactivity was detected in all animals. In 100% of infected animals presenting LGIEN, HGIEN or adenocarcinoma, gradual decrease of the intensity of cytoplasmic *Apc* labeling after infection with diverse *C. parvum* strains was recorded in the lesions, while contiguous normal mouse tissue showed a staining pattern similar to that seen in normal tissue (Fig. 1). The decrease of *Apc* labeling was observed after 25 days P.I and was found in lesions with either intraepithelial neoplasia or invasive adenocarcinoma (Table 1). An association between loss of *Apc* labeling and a longer time P.I. was extremely significant ($p<0.001$). The incidence of this altered decrease of labeling was higher in animals with higher parasite amounts in tissues ($p< 0.001$).

β-catenin and E-cadherin labeling

In the mucosa of the ileo-caecal regions of uninfected control mice, β-catenin labeling was light and exclusively localized in the cell membrane. In 6/11 (55%) *C. parvum* infected mice presenting LGIEN or HGIEN, and in 5/6 (83%) presenting adenocarcinoma there was a

progressive increase of the cytoplasmic labeling after 25 days P.I. without loss of β-catenin membrane labeling. We did not observe nuclear β-catenin labeling. β-catenin staining was more extensive in the cytoplasm of the cells with more severe lesions (Fig. 2A, 2B). The alteration of β-catenin expression was significantly associated to a longer time P.I. and to higher amounts of parasites in tissues ($p< 0.001$ and $p< 0.001$, respectively). However, this alteration of β-catenin expression was not associated with the score of severity of the neoplastic lesions (Table 1).

In order to confirm our observations about the localization of β-catenin, more experiments were conducted. We performed immunofluorescence (IF) analysis using 2 different antibodies, one directed against the β-catenin C-terminus and the other against the N-terminus. We noticed a membranous and juxta-membraneous localization of β-catenin in ileo-caecal tumor regions obtained from infected animals presenting an invasive adenocarcinoma after 90 days P.I.. This accumulation of β-catenin was observed mainly at a basolateral position. The absence of β-catenin in the nucleus of ileo-caecal tumor regions after 90 days P.I. was confirmed (Fig. 2C, 2D).

In addition, Western Blot analysis was performed using the antibodies mentioned above. Firstly, on total cellular tumor lysates, an increase in β-catenin expression was observed (Fig. 2E). Secondly, after fractionation of epithelial cells of tumors, traces of nuclear β-catenin (F3 fraction) were observed in both, control and *Cryptosporidium*-infected mice but with the same degree of protein expression. In addition, an increase of β-catenin was found in the F2 fraction (membrane and organelles) of *Cryptosporidium*-infected mice when compared to control animals (Fig. 2F), and this difference was stronger using the anti-β-catenin N-terminus antibody.

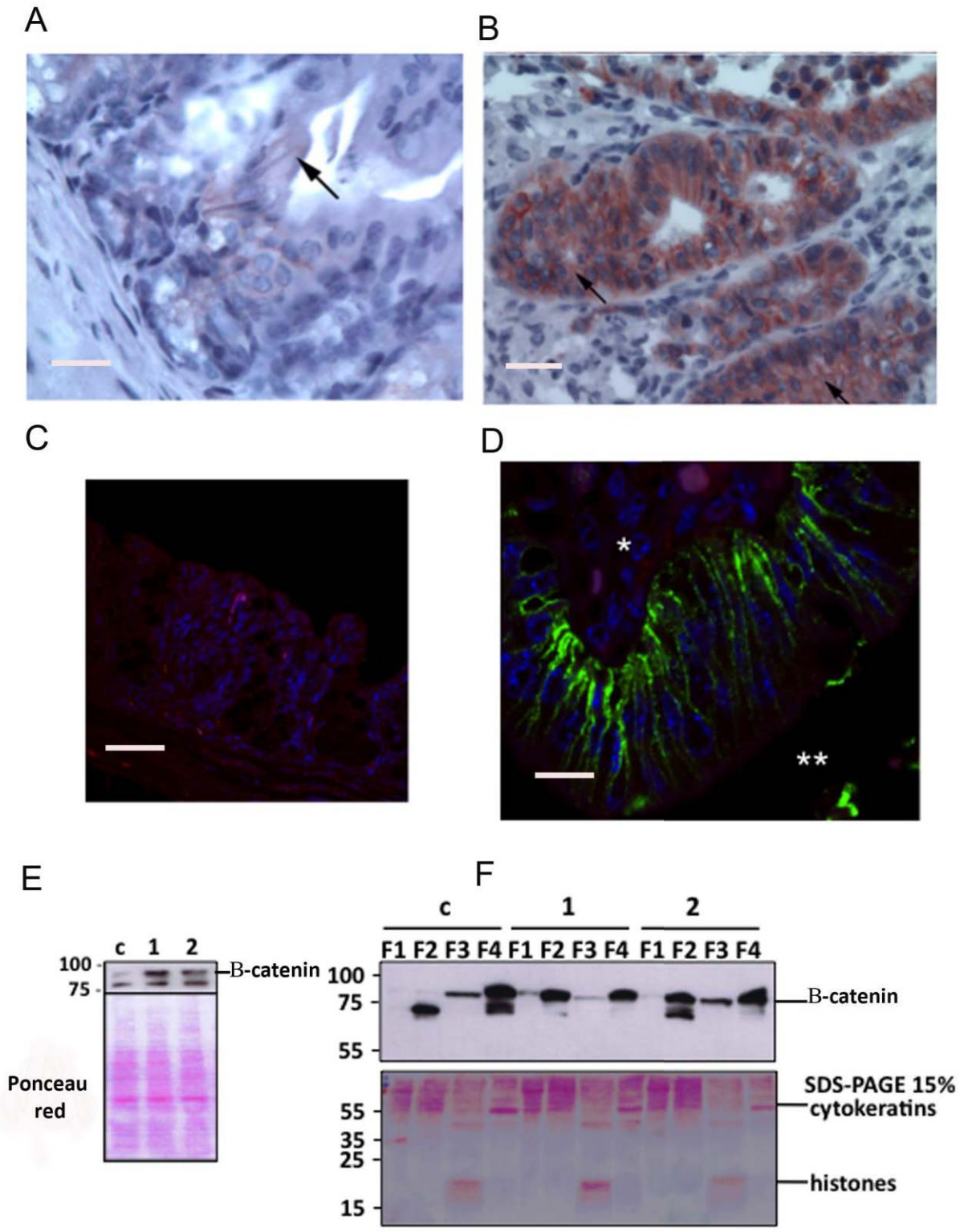
As well, a reduced labeling of the protein E-cadherin in the host cell membrane was observed associated to neoplastic lesions of the ileo-caecal region of *C. parvum* infected animals after 45 days P.I.

Table 1. Summary of immunohistochemical staining results according to the day post infection, the amount of parasite and severity of ileo-caecal region lesions

	***B*-catenin abnormal expression**	***p* value**	***APC* abnormal expression**	***p* value**	**P53 abnormal expression**	***p* value**
Days Post-infection						
< 25 days	0/6 (0%)	**0.0004**	0/6 (0%)	**0.0001**	0/6 (0%)	**0.03**
> 25 days	17/20 (85%)		10/10 (100%)		5/8 (62.5%)	
Amount of parasites[a]						
< +3	0/7 (0%)	**0.0001**	3/6 (50%)	**0.04**	1/4 (25%)	0.55
> +3	13/13 (100%)		9/9 (100%)		6/10 (60%)	
Score of severity of lesion[b]						
Low or high grade intraepithelial neoplasia	6/11 (54.5%)	0.33	8/8 (100%)	1	2/5 (40%)	0.19
Adenocarcinoma	5/6 (83.3%)		5/5 (100%)		3/3 (100%)	

[a] 0, no parasites; +1, small number of parasites, focally distributed; +2, moderate number of parasites, widely distributed; +3, abundant parasites present, widely distributed throughout the section.

[b] 0, no lesion; 1, inflammation and/or regenerative changes; 2, low-grade intraepithelial neoplasia (LGIEN); 3, high-grade intraepithelial neoplasia (HGIEN). In this category, carcinoma in situ (limited to the epithelium), and intramucosal adenocarcinoma (invasion into the lamina propria) were also included; 4, invasive adenocarcinoma (penetration of neoplastic glands into the submucosa); 5, adenocarcinoma with invasion into the muscularis and deeper.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55
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55
35
25
15
SDS-PAGE 15%
cytokeratins
histones

Fig. 2. Detection of Beta catenin cellular localization by immunohistochemistry, immunofluorescence and Western Blot analysis. (A) Section of a normal non-neoplastic mucosa of the ileo-caecal region showing a light membranous expression (arrow) after immunostaining for β-catenin. Bar= 10 µm. (B) Ileo-caecal epithelial cells from a *C. parvum* infected mouse with invasive adenocarcinoma and euthanatized at 107 days P. I. show, after immunostaining for β-catenin, a persistent membranous expression along with a prominent cytoplasmic one(arrow). Bar= 15 µm. (C) Section of a normal non-neoplastic mucosa showing absence of β-catenin expression after immunofluorescence using anti β-catenin antibody directed to the C terminus. Bar= 5 µm. (D) Ileo-caecal epithelial cells from a *C. parvum* infected mouse with invasive adenocarcinoma, euthanatized after 90 days P.I. show membranous and juxtamembranous expression of β-catenin at a basolateral position after immunofluorescence using an anti β-catenin antibody directed to the C-terminus. Bar= 25 µm, *= basal membrane, **= lumen of glands. (E) Western Blot analysis using an anti β-catenin antibody directed to the N-terminus: decrease in β-catenin expression in total cellular lysates from normal non-neoplastic mucosa of the ileo-caecal region of non-infected Dex-treated SCID mouse (c); increase in β-catenin expression in total cellular tumor lysates from *C. parvum* infected mice euthanatized at 90 days P. I.. (F) Western Blot using an anti β-catenin antibody directed to the N-terminus after fractionation of epithelial cells : traces of nuclear β-catenin (F1) were observed in both, control (c) and *Cryptosporidium*-infected mice (1) and (2) with the same degree of protein expression. Increase of β-catenin was found in the F2 fraction (membrane and organelles) of *Cryptosporidium*-infected mice. F1: cytosol; F2: membranes and organelles, F3: nuclei; F4: cytoskeleton.

***Kras* labeling**

We did not find differences in *Kras* labeling in both ileo-caecal epithelia from negative controls and *Cryptosporidium* infected mice. At any moment of the evolution of the infection or the neoplastic lesions a normal membrane staining was observed.

p53 labeling

All animals failed to show nuclear expression for p53 in the adenomatous ileo-caecal region. Cytosolic p53 labeling in the adenomatous cells was observed in 5/8 (63%) mice infected with *C. parvum* after 25 days P. I.. The alteration of the expression of p53 was significantly associated with the time P.I. (p=0.03) but not with the amount of parasites in tissues or the severity of the neoplastic lesions (Table 1).

***Apc* β-catenin, and *Kras* mutations in tumors**

In total, non-neoplastic intestinal tissue from non-infected SCID mice and 5 polypoid adenomas from four *C. parvum* infected mice were analyzed for 3 genes: *Apc, β*□catenin and *Kras*, using high-throughput sequencing. The analysis of data showed that in *C. parvum* infected and non-infected SCID mice there were the same single nucleotide polymorphisms (SNP), as showed in table 2.

However, using a range of depth of 50X we did not find allelic variations in the regions of *Apc*, β-catenin and *Kras* considered most likely to have mutations associated to colorectal cancer.

Table 2. Data resulting from the analysis of selected regions of three genes *APC, β-catenin* and *Kras* in non-infected and infected SCID mice tissue using high-throughput sequencing.

Loci	Reference position	Variation type	Length	Reference	Variants	Allele variation	Quantity	Depth	Frequency (%)
APC-A	397	SNP	1	A	1	T	3778	3810	99.2
APC-F	233	SNP	1	A	2	A/C	71/50	128	55.5/39.1
Kras	100	SNP	1	T	1	C	322	322	100
Kras	182	SNP	1	T	1	C	108	119	90.8
Kras	388	SNP	1	T	1	C	419	419	100

Electron Microscopy

A dilation of intercellular spaces with extensive development of lateral membrane extensions was observed at the level of adherens junctions in the antro-pyloric and ileo-caecal neoplastic epithelia of *C. parvum* infected mice (Fig. 3). Mice infected with *C. muris,* species with gastric tropism and not associated to digestive neoplasia, were analyzed to establish a comparison with *C. parvum*, and alterations in ultrastructure of intercellular junctions of *C. muris* infected epithelial cells were not found.

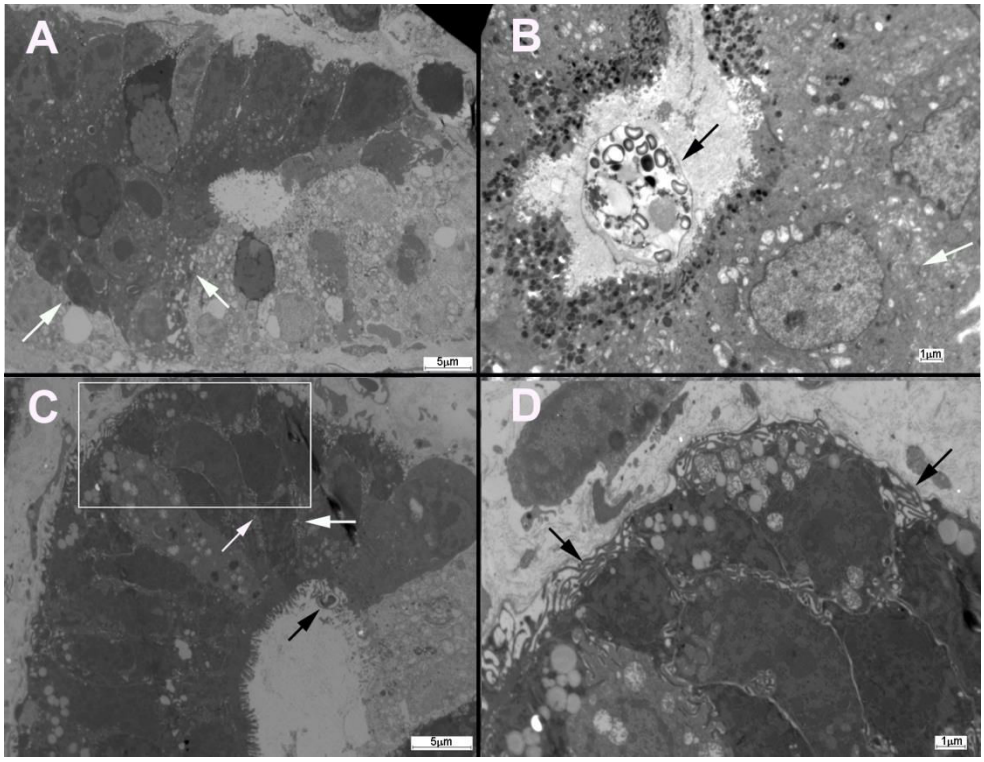

Fig. 3. (A) Electron micrograph of a section of a normal non-neoplastic mucosa showing normal intercellular junctions (white arrows) (B) In SCID mice infected with *C. muris* (black arrow), alterations in ultrastructure of intercellular junctions (white arrow) of gastric epithelial cells were not found. (C) A dilation of intercellular spaces with extensive development of lateral membrane extensions (white arrows) was observed at the intercellular junctions of the ileo-caecal epithelia of *C. parvum* (black arrow) infected mice. (D) Detail of C showing lateral membrane extensions (white arrows).

DISCUSSION

In the present study using our rodent model of Dex treated SCID mice, we demonstrate for the first time that Wnt signaling pathway is implicated during the development of *C. parvum* induced neoplastic process independently of the inoculated strain. This process seems to be atypical considering that unlike colorectal adenocarcinomas, mutations in selected loci from crucial genes belonging to this signaling pathway (*Apc* and *β*-catenin) were not found, even if alterations in the expression of the immunostaining pattern were observed.

In our study, a gradual decrease of the intensity of cytoplasmic *Apc* labeling after infection with diverse *C. parvum* strains was recorded associated to neoplastic lesions of the ileo-caecal region. *Apc* encodes a 2843 amino acid cytoplasmic protein, whose one of the main functions is to facilitate the ubiquitination and destruction of β-catenin having a link with the Wnt signaling pathway (Kuraguchi et al., 2000). Nearly all of the tumor-associated somatic mutations in human *Apc* occur within the first 1500 codons and approximately two-thirds are confined to a mutation cluster region between codons 1286 and 1513. When these mutations are present a truncated *Apc* protein is unable to down-regulate β-catenin. As a consequence, cytosolic levels of β-catenin are elevated and the protein can entry into the nucleus, where it can mediate transcriptional regulation. With the discovery of the association of *Apc*'s Arm repeats to proteins involved in cytoskeletal dynamics, another important functional role of *Apc* in cell morphology and migration through modifications of the actin cytoskeleton became apparent (Buda and Pignatelli, 2004,Giles et al., 2003).

Using high-throughput sequencing, we examined in detail the regions of *Apc* considered most likely to have mutations in intestinal tumors in humans and mice between codons 789 and 1464 (Kuraguchi et al., 2000).While it is well known that most human and rodent intestinal tumors contained mutations in these regions, in our study mutations were undetectable by the highly performing methods we used. In a model of C57BL/6J Min/+ mice it was found that *Apc* truncation of its carboxy C-terminus was associated with reduced enterocyte migration and alteration of adherens junction proteins, and it was hypothesized that these defects resulting from truncation of the *Apc* C-terminus which contains microtubule binding regions and putative sites for indirect actin binding were related to changes in cytoskeletal function (Hughes et al., 2002). Then, it is possible that *C. parvum*-induced tumors may contain truncation mutations in *Apc* outside the tested region.

In some cases, tumorigenesis is initated by alterations in molecules other than *Apc*. β-catenin is a 92 kDa protein that together with E-cadherin plays a role in cell–cell adhesion and is involved in intracellular signaling. Colorectal adenomas and carcinomas show translocation of β-catenin from the cell membrane to the cytoplasm/nucleus, and this event is considered as an early event in the development of colorectal neoplasia (Takahashi et al., 2000). In addition, mutational activation of β-catenin has been described in some instances of human colorectal cancer and this may bypass *Apc* mutations. In rats, cytoplasmic/nuclear translocation of β-catenin has been reported in azoxymethane-induced adenomas and carcinomas, and in this model impairment of *Apc* function is rare (Cooper et al., 2000). In our analysis, we found an abnormal juxtamembranous localization of β-catenin in 100% of mice with intraepithelial neoplasia or adenocarcinoma but we never observed nuclear expression of β-catenin as confirmed by three different technics. Consistently, in transformed lymphocytes infected with Epstein Barr Virus it has been reported that the increase of β-catenin was not nuclear (Everly et al., 2004).

In addition to the alteration of β-catenin labeling, a reduced expression of the transmembrane protein E-cadherin was observed at the cell membrane of ileo-caecal epithelia after 45 days P.I.. E-cadherin forms the key functional component of adherens junctions of colonic epithelial cells. E-cadherin binds catenins (α, β, and γ) to form cytoskeletal complexes required for maintenance of epithelial cell polarity, preserve barrier function and intercellular adhesion (Buda and Pignatelli, 2004). Abnormalities in the cadherin/catenin complex as we observed, could result in reduced cell-cell adhesion and conversion to a migratory phenotype.

Nevertheless, we did not find oncogenic β-catenin mutations in tumor samples of mice that lack *Apc* mutations. Additionally, transcriptional silencing of *Apc* by promoter methylation has been suggested as an alternative to somatic mutation (Segditsas and Tomlinson, 2006). Another study revealed the presence of concurrent methylation of groups of genes in hepatocellular carcinoma associated with hepatitis B virus and hepatitis C virus infections, suggesting that aberrant epigenetic changes associated with viral infection and exposure to environmental factors may activate events that promote the neoplastic transformation of hepatocytes (Lambert et al., 2011).

The role of p53 in human colitis-associated neoplasia has been studied by both immunohistochemistry and molecular techniques and it has been reported that nuclear expression and presence of mutations of this gene can occur as early events in contrast to non-colitic colorectal neoplasias (Cooper et al., 2000). In our study, we found accumulation

of p53 only in the cytoplasm of infected mice after 25 day P.I.. Thus, the p53 signaling pathway appears to be involved in the process. These observations are consistent with another study reporting that *Theileria annulata* schizont leads to cytoplasmic sequestration of the majority of host cell p53, resulting in the inhibition of p53-mediated apoptosis and promotion of host cell survival (Haller et al., 2010). Additionally, cytosolic accumulation of p53 in human colon cancer due to sequestration of the protein by the actin filaments has been described (O'Brate and Giannakakou, 2003).

We did not examine the presence of possible mutations in p53 due to the fact that p53 genetic alterations in murine epithelial cells could be lower than those seen in humans. In fact, carcinogen-induced tumorigenesis in colorectal cancer in both mice and rats has resulted in conflicting reports regarding molecular alterations and nuclear expression of p53 with some authors reporting no role while others reporting a role for p53 (Cooper et al., 2000).

Along the known sequence of colonic adenoma to carcinoma, loss of *Apc* function is usually followed by oncogenic activation of *Kras* (Janssen et al., 2006). *Kras* encodes an intracellular signaling molecule and its alteration results in constitutive alteration of Ras and its down-stream signaling pathways, playing an important role in cell death, differentiation and proliferation. Point mutations in *Kras* are among the most frequent genetic alterations in colorectal cancer in humans, and also they have been described in rodent models (Hu et al., 2009). After analysis of the proto-oncogene *Kras* we did not find alterations in its expression or at genomic level. However, some SNP located in non-coding regions of the ADN of *Apc* and *Kras* were detected either in neoplastic or non neoplastic tissue samples. After analysis it was found that these variations have not been reported before in association to cancer. However, further exploration of this pathway may be performed in a future research work.

Based on the alterations we found for *Apc* and β-catenin expression, the Wnt pathway seems to be implicated in *C. parvum*-induced ileo-caecal adenocarcinoma. Furthermore, in neoplastic lesions, a stabilization of β-catenin, an abnormal accumulation at a basolateral position and a dilation of intercellular spaces with extensive development of lateral membrane extensions at the level of adherens junctions were observed. As described by other authors, these results may be interpreted as β-catenin recruitment to membrane ruffles (lamellipodia) implicated in the transformed cells migration (Johnson et al., 2013,Odenwald et al., 2013).

Consistently, β-catenin was not observed in the nucleus of epithelial cells. Then, we hypothesized that there is also an activation of the non-canonical WNT pathway which involves the Rho GTPase signaling without β-catenin gene transactivation. For instance, it has been described that Rho GTPases and Wnt signaling are highly interconnected pathways that can influence hepatocarcinogenesis (Lechel and Rudolph, 2008). Furthermore, it is well known that *Cryptosporidium* infection induces cytoskeletal changes that modulate a localized actin reorganization and channel/transporter insertion, and it is likely that the signaling events initiated at the interface between host and pathogens induce whole cell, and perhaps tissue-level changes in the cytoskeletal architecture. These signaling axes include the phosphatidylinositol-3-kinase (PI3K), the guanine exchange factor, Frabin-dependent activation of the small GTPase, CDC42 and c-Src -dependent activation of cortactin (O'Hara and Chen, 2011).

In conclusion, the present results indicate that *C. parvum* independently of the strain is able to modulate host cytoskeleton activities and several host-cell biological processes that could explain the transformed phenotype of infected epithelial cells. However, it is still unclear which could be the specific cellular transformation induced by *Cryptosporidium*. It is possible that a combination of several pathways is needed to transform infected cells. Then, we have also to consider the exploration of other signaling pathways in the future. It is of particular interest to investigate whether alteration in Rho GTPase pathway may contribute to colonic carcinogenesis.

How the cell senses the pathogen and adjusts its transcription and translation programs to its new life with a parasite remains an important issue (Cossart and Sansonetti, 2004). Furthermore, reports suggesting an association of cryptosporidiosis with cancer in humans together with our experimental observations described herein, largely justify the development of research on the topic, and incite to use this original animal model to approach the subject.

MATHERIAL AND METHODS

Cryptosporidium parvum oocysts

Oocysts of *C. parvum* IOWA and *C. muris* RN66 (purchased from Waterbo*rne*™, New Orleans, Louisiana), *C.parvum* TUM1 (kindly given by Dr. D. Akiyoshi and Dr. S. Tzipori from Tufts Cummings School of Veterinary Medicine, USA) and *C. parvum* II2A15G2R1 (strain isolated from stools of an immune depressed patient who developed fulminant cryptosporidiosis after near-drowning in Lille) were used. The stock solutions of oocysts

were stored in a conservation medium (phosphate-buffered saline or PBS with penicillin, streptomycin, gentamycin, amphotericin B and 0.01% Tween 20) at 4°C until use. Before inoculation, absence of other pathogens in the inoculum was excluded by plating it into selective or nonselective culture media (Trypticase soy, Trypticase soy and blood, Hektoen, Tergitol 7 with TTC, Difco *Pseudomonas* Isolation Agar, Saboureaud). Oocyst viability was assessed by testing excystation (Certad et al., 2010b,Certad et al., 2007).

Experimental hosts

Seven-week-old CB17-SCID mice were obtained from a colony bred at the Pasteur Institute of Lille (France) and regularly controlled for assessing unwished microbial (including *Helicobacter*), or parasitological pathogens. Animals were housed in groups in covered cages and maintained under aseptic conditions in an isolator with standard laboratory food and water ad libitum.

Experimental design

SCID mice were administered with 4 mg/L of dexamethasone sodium phosphate (Dex) (Merck, Lyon, France) via drinking water. Dex administration started two weeks prior to oral inoculation with *Cryptosporidium* oocysts and was maintained during the whole experimentation. Dex-added water was replaced three times a week.

For immunohistochemical and genetical studies infective doses of 10^5 oocysts per mouse of three different strains of *C. parvum* were prepared as previously (Certad et al., 2012,Certad et al., 2010a,Certad et al., 2010b,Certad et al., 2007) and were inoculated by oral-gastric gavage to 27 animals (IOWA: 20 mice, TUM1: 4 mice and IIaA15G2R1 human isolate: 3 mice). For ultrastructural studies, infective doses of 10^5 oocysts of *C. muris* per animal were similarly prepared and inoculated. In order to localize β-catenin, 7 additional animals were inoculated and followed 90 days P.I. In total, twelve negative control mice were inoculated only with PBS.

In order to determine parasite shedding fecal specimens were collected and processed as previously described (Certad et al., 2010b). Periodically or when signs of imminent death appeared, mice were euthanatized by carbon dioxide inhalation. Assessment of animal's clinical condition was performed regularly to detect and then minimize suffering. Clinical signs that could constitute an endpoint included, but were not limited to: rapid or progressive weight loss, debilitating diarrhea, rough hair coat, hunched posture, lethargy or any condition interfering with daily activities (e.g. eating or drinking, ambulation, or elimination).

Experiments were conducted in the animal facility of the Institut Pasteur de Lille (research accreditation number, A59107). Animal protocols were approved by the French regional ethical committee (approval number CEEA 112011).

Histopathology and immmunohistochemistry

Ileo-caecal regions were removed, fixed in 10% buffered formalin and processed using standard staining techniques (H & E). Formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 5 µm. Then all sections were deparaffinized, rehydrated through serial dilutions of alcohol, and washed in phosphate-buffered saline (pH 7.2). To assess and score the level of invasion of the neoplastic process different techniques were used as previously. The Volgens-Gomori stain (Bulckaen et al., 2008) was employed for assessing the gland basement membrane integrity. An anti-cytokeratin monoclonal antibody (AM071-5M, Biogenex, Netherlands) was used to evaluate the invasion of epithelial cells into the lamina propria and in deeper organ layers. Anti-alpha smooth muscle actin monoclonal antibody (dilution 1:100) (M0851, Dako, Denmark) was used to stain muscle fibers in order to visualize muscularis mucosae disruption or the muscularis penetration by neoplastic glands.

Other immunohistochemistry techniques were performed to explore metabolic alterations that could be involved in the neoplastic process: anti-β-catenin (H-102): sc-7199, Santa Cruz Biotechnology, Inc, Dallas, U.S.A.) rabbit polyclonal antibody (1/125) directed to the C-terminus, anti-*Apc* rabbit polyclonal antibody (Sc-896, Santa Cruz Biotechnology, Inc. Dallas, U.S.A.)) (1/100), anti-p53 rabbit polyclonal antibody (ab4060, Abcam Cambridge, U.K) (1/50), that reacts with both wild-type and mutant p53, anti E-Cadherin mouse anti-human antibody (NCH-38) (1/50) and anti-*Kras* rabbit polyclonal antibody (12063-1-AP, Protein Tech, Chicago, U.S.A.) (1/100) were used. Sections were immersed in pre-heated (95-100°C) citrate buffer (pH 6.0) for β-catenin, P53 and *Kras* antibodies, and in EDTA buffer (pH 8.0) for the *Apc* antibody**.** For E-Cadherin staining the sections were treated with Ag retrieval, rinsed in TBS buffer. Endogenous peroxydase activity was blocked using 3% hydrogen peroxide solution for 10 minutes. Then samples were incubated with antibodies for 60 min at 25 °C: the following dilutions were applied: Conventional biotine-streptavidin peroxidase was performed, and the slides were counter-stained with hematoxylin. Controls for marker expression were normal tissues from non infected mice or non neoplastic regions of the same slide. Human colorectal and mammary adenocarcinomas were used respectively as positive controls for p53 and *Kras* antibodies. Sections were examined by two pathologists

using a Leica DMRB microscope equipped with a Leica digital camera connected to an Imaging Research MCID analysis system (MCID software, Cambridge, UK).

Parasite load in digestive sections was scored on 5 selected fields at a magnification of 400 X, as described before (Certad et al., 2010a,Certad et al., 2010b). Histopathological lesions at different sites were scored as previously (Certad et al., 2010a,Certad et al., 2010b) with slight modifications. Briefly: 0, no lesion; 1, inflammation and/or regenerative changes; 2, low-grade intraepithelial neoplasia (LGIEN); 3, high-grade intraepithelial neoplasia (HGIEN). In this category, carcinoma in situ and intramucosal adenocarcinoma were also included; 4, adenocarcinoma invading the submucosa); 5, adenocarcinoma with invasion into the muscularis and deeper.

Confocal immunofluorescence

Ileo-caecal regions were removed, fixed in 10% buffered formalin and embedded in paraffin. Five µm thick sections were placed on glass slides and deparaffinized using graded ethanol. This progressive rehydration was followed by an antigen retrieval step using citrate buffer pH 6.5 in a microwave oven for 15 min. After 30 min blocking with PBSG buffer (phosphate buffer saline, 1.2 % glycine, pH 7.3), the primary antibodies were applied diluted in PBSG for 1 hour at 37°C. After a 3 X 5 min wash in the same buffer, the specimens were incubated in the secondary antibodies at the same conditions. After a final wash, DAPI was added to stain the nuclei for 10 min and the sections were mounted in an antifading solution (Mowiol). The visualization was achieved on a Zeiss LSM780 confocal microscope: the 405 nm, 488 nm and 545 nm laser lines were used respectively for DAPI, AlexaFluor 488 and Alexa Fluor 545 visualization. The same instrumental settings (laser power, scan speed) were applied throughout the experiment to be able to compare the labeling of the different specimens. The following primary antibodies were used: anti-ß-catenin ((H-102): sc-7199, Santa Cruz Biotechnology, Inc, Dallas, U.S.A.) rabbit polyclonal antibody (1/125) directed to the C-terminus, and anti-β-catenin ((E247): ab32572, Abcam, Cambridge, UK) rabbit monoclonal antibody (1/125), directed to the N-terminus. The secondary antibodies used were: Alexa Fluor 488–conjugated antirabbit IgG antibody (1/500; Molecular probes, Life technologies, Carlsbad, U.S.A.) and AlexaFluor 545-conjugated anti-mouse IgG antibody (3/1000; Molecular probes, Life technologies, Carlsbad, U.S.A.)

Fractionation of epithelial cells and Western Blot analysis

Visible tumours were collected from mice euthanatized at day 90 PI and processed to perform a fractionation of epithélial cells following the instruction of the ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem, Merck Millipore, Molsheim, France) kit. For each fraction (F1 to F4) protein concentration was mesured using micro BCA protein assay kit (Pierce, Fisher Scientific, Illkirch, France) Proteins (30 µg) were separated by SDS-PAGE, and Western blot analysis was carried out as previously described (Olivier-Van Stichelen et al., 2012) using two different anti-β-catenin antibodies, anti-β-catenin ((H-102): sc-7199, Santa Cruz Biotechnology, Inc, Dallas, U.S.A.) rabbit polyclonal antibody (1/5000) directed to the C-terminus, and anti-β-catenin ((E247): ab32572, Abcam, Cambridge, UK) rabbit monoclonal antibody (1/5000), directed to the N-terminus.

Ultrastructural study

Fixation and epon-embedding procedures of lesions identified macroscopically at the ileo-caecal region for *C. parvum* infected mice and at antro-pyloric region for both *C. parvum* and *C. muris* infected mice were carried out fixing in glutaraldehyde 2.5% solution and washing in phosphate buffer solution (0.1 M, pH 7.4) that optimized the preservation of cell structures (Aliouat et al., 1995,Palluault et al., 1992). Ultra thin sections were contrasted with uranyl acetate and lead citrate and examined using transmission electron microscope (LEO-906, Leica, Rueil-Malmaison, France).

High-throughput sequencing

In total, 5 polypoid visible lesions and antropyloric regions from four *C. parvum* infected mice at day 90 P. I. were dissected. Additionally, normal tissue samples were obtained from non-infected control mice. Genomic DNA was prepared immediately using the NucleoSpin tissue (Macherey-Nagel, Düren, Germany).

The regions of *Apc* (Kuragushi et al., 2000), *β*-catenin and *Kras* (Takahashi et al., 2000; Takahashi & Wakabayashi, 2004) considered most likely to have mutations associated to colorectal cancer in human and mice were selected for primer design. Table S1 lists corresponding primer pairs for 16 amplicons representing *Apc*, *β*-catenin, and *Kras*.

PCR protocols were performed with genomic DNA amplified in 100 µl reactions containing 2 mM $MgCl_2$, 250 nM primers, 250 µM dNTP, 5 U HotStart Taq (Qiagen). PCR reactions were performed using a 96-Well MasterCycler EP, Eppendorf PCR System. Cycle conditions

included initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C during 20 s, annealing at 58°C for 15, extension at 72°C during 45 s. and the reaction ended with a last extension step at 72°C during 4 min.

After purification and quantification, the resulting amplicon products were pooled in an equimolar manner and barcode adaptor sequences were incorporated. The monitoring of barcoded library was done by BioAnalyzer (Agilent Technologies). Then, all the samples were pooled in an equimolar manner and finally, the library was amplified by emPCR to obtain sequencing template. The sequencing was performed according to the Ion PGM 200 Sequencing Kit (Life technologies) and the data analysis was performed using CLC genomics workbench 5 modules.

Table S1. List of corresponding primer pairs for 16 amplicons representing Apc, β-catenin, and Kras

Name	Sens	ID GenBank, position	Sequence	Length	Tm(°C)
APC A	forward	NC_000084.6: 34312278-34312296	tcccggctcaagtctgcca	19	60,7
APC A	reverse	NC_000084.6: 34312690-34312709	gctatctgggctgcagtggt	20	59,4
APC B	forward	NC_000084.6:34312689-34312709	taccactgcagcccagatagc	21	58,7
APC B	reverse	NC_000084.6: 34313042-34313062	gggctaggtcagctggatact	21	57,8
APC C	forward	NC_000084.6: 34313041-34313062	cagtatccagctgacctagccc	22	58,8
APC C	reverse	NC_000084.6: 34313254-34313278	agacaggataactggtgttctggct	25	59.1
APC D	forward	NC_000084.6: 34313254-34313278	agccagaacaccagttatcctgtct	25	59.1
APC D	reverse	NC_000084.6: 34313734-34313756	gctgaacttggacgcagctgatt	23	60
APC E	forward	NC_000084.6: 34313734-34313756	aatcagctgcgtccaagttcagc	23	60
APC E	reverse	NC_000084.6: 34314176-34314195	gagcggagtctcctggacat	20	58.1
APC F	forward	NC_000084.6: 34314176-34314199	atgtccaggagactccgctcgtat	24	60.5
APC F	reverse	NC_000084.6: 34314466-34314485	accctctgcacggcagcatt	20	61.6
Kras	forward	forward NC_000072.6: 145247097-145247077	cctttgagagccattagctgc	21	56.2
Kras	reverse	NC_000072.6: 145246708-145246687	agcgttacctctatcgtagggt	22	56.2

beta-cat	forward	NC_000075.6: 120950465-120950490	cgtagatggcttcttcaggtagcatt	26	58.2
beta-cat	reverse	NC_000075.6: 120950787-120950807	gctgtcacacagccctgtcaa	21	59.7

Statistical analysis

Fisher's exact test (two-tailed) was used. Data analysis was performed with the statistical software Graphpad. Significance was defined as $P< 0.05$.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: S.B., V.C, M.C., M.P., C.A., K.G., E.V., C.C., E.D. C.Cr., G.C. Performed the experiments: S.B., V.C., M.C., C.A., R.B., K.G., S.G., A.M., T.C., B.D., N.G., V.D., C.S., G.C. Analyzed the data: S.B., V.C., M.C., M.P., C.A., K.G., S.G., A.M., T.C., M.O., V.D., T.L., C.S., E.V., C.C., E.D.C., C.Cr., G.C. Contributed reagents/materials/analysis tools: A.M., T.C., E.V., C.C. Wrote the paper: S.B., C.A., M.P., C.Cr., E.D.C ., G.C. All authors approved the content of the manuscript.

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VII. *Discussion*

Axe 1 : Premières données d'épidémiologie moléculaire et facteurs de risque liés à l'infection par *Cryptosporidium* spp. au Liban

Cryptosporidium est un protozoaire opportuniste pathogène très largement répandu dans le monde et qui a pris une grande importance en clinique humaine et vétérinaire dans les 30 dernières années du fait de sa pathogénicité chez les hôtes immunodéprimés ou les enfants. Il est responsable de diarrhées spontanément résolutives chez les patients immunocompétents, notamment les enfants, et de diarrhées chroniques graves chez les malades immunodéprimés (Guyot et al., 2012; Hunter and Nichols, 2002; Ryan and Hijjawi, 2015).

Malgré son intérêt évident en santé publique, aucune information n'était disponible concernant la situation actuelle de la cryptosporidiose dans un pays comme le Liban. En effet, seules deux études, réalisées sur un faible effectif de patients sidéens libanais et en utilisant des techniques peu sensibles, ont décrit une prévalence allant de 3% à 50% dans cette population (Boujaoude et al., 2000; Naba et al., 2010). Aussi, nous avons voulu dresser ce qui est à notre connaissance le premier portrait de l'épidémiologie moléculaire de l'infection par *Cryptosporidium* spp. au Liban, plus particulièrement dans le Nord du pays.

Le Liban, à l'instar de la plupart des pays en développement, est très touché par les infections parasitaires intestinales. Hamze *et al* ont rapporté en 2004 après analyse par examen macroscopique puis microscopique des selles, une prévalence de 33% d'infections parasitaires dans une population totale de 17 126 patients hospitalisés (Hamze et al., 2004). D'autre part, une autre étude du même groupe de 2008 a révélé une prévalence de 58% de parasitoses digestives dans une population de 308 adultes travaillant dans un secteur alimentaire (Hamze et al., 2008). Cependant, *Cryptosporidium* spp. ne fut pas recherché dans ces deux études. Même si ces données ont été basées sur une technique d'identification présentant une faible sensibilité en l'occurrence sur l'observation microscopique de selles, elles ont pourtant confirmé une forte prévalence des infections parasitaires due probablement aux problèmes d'hygiène personnel, de pollution de l'eau potable et à l'absence d'un système de surveillance et de control des parasitoses intestinales dans ce pays.

Dans ce contexte, nous avons décidé, dans un premier temps, de déterminer la prévalence de la cryptosporidiose, en se focalisant sur l'étude d'une population de patients symptomatiques suivis pour différentes pathologies dans plusieurs hôpitaux du Nord-Liban (Article 1).

Cryptosporidium a été identifié par la coloration de Ziehl Neelsen modifiée dans 6% des échantillons de selles testés (10/163 patients). Or, cette méthode d'identification est comparativement moins sensible que la PCR nichée (Jex et al., 2008). Nous avons d'ailleurs

trouvé une prévalence de 9.2% de cryptosporidiose dans la même population (15/163 patients) après une analyse moléculaire ciblant l'ADNr 18S. (Article 1)

Dans un deuxième temps, nous avons cherché à caractériser la prévalence de *Cryptosporidium* ainsi que d'autres protozoaires intestinaux (*Blastocystis* sp, *Dientamoeba fragilis* et *Giardia duodenalis*) dans une cohorte d'enfants de 3 à 16 ans fréquentant deux écoles de Tripoli de niveaux socio-économiques différents. Cette étude nous a permis aussi d'identifier les potentiels facteurs de risques associés aux infections parasitaires intestinales (Article 2). La population des enfants est une des populations la plus susceptible à la cryptosporidiose (Striepen, 2013) ce qui pourrait être lié à différents facteurs comme une immunité déficiente et des carences alimentaires surtout chez les enfants âgés de moins de 2 ans, la promiscuité et surtout un faible niveau d'hygiène dans les pays en développement. Tous ces facteurs facilitent la transmission des parasites (Alyousefi et al., 2011; Tulu et al., 2014)

Après analyse des échantillons, *Blastocystis* sp. était le parasite le plus prévalent (63%), suivi par *Dientamoeba fragilis* (60,6%), *Giardia duodenalis* (28,5%) et *Cryptosporidium* spp. (10,4%). Comme attendu, la prévalence de ces protozoaires était plus faible après détermination par observation microscopique des montages humides (51,6%, 14,4%, 5,6% et 0%, respectivement). D'autres parasites intestinaux ont également été détectés par examen microscopique, tels que *Entamoeba histolytica / dispar* (5,6%), *Entamoeba coli* (2,4%), *Ascaris lumbricoides* (0,4%), et *Hymenolepis nana* (0,4%). Par contre, pour des raisons logistiques, des techniques spécifiques pour le diagnostic de certains nématodes n'ont pas été utilisées, tel que le Scotch-test pour la détection d'*Enterobius vermicularis* (Maguire, 2014).

Des infections mixtes avec deux parasites ont été trouvées chez 35,7% des enfants (89/249). La double infection la plus fréquente combinait *Blastocystis* sp. et *Dientamoeba fragilis*, avec une prévalence de 68,5% (61/89). En outre, un certain nombre d'enfants présentaient des infections parasitaires triples ou quadruples. Dans les études précédentes (Hamze et al., 2004; Hamze et al., 2008), *Entamoeba coli*, *Ascaris lumbricoides* et *Giardia duodenalis* étaient les parasites prédominants retrouvés dans la population libanaise. De ce fait, nos données différaient de celles obtenues dans ces études de 2004 et 2008. Pour expliquer ces différences, on doit rappeler que dans les études précédentes, *Blastocystis* sp., *Dientamoeba fragilis* et *Cryptosporidium* spp. n'avaient pas été ciblés et que nous avons utilisé, dans notre présente analyse, de techniques de diagnostique plus sensibles.

Les résultats de ce travail de thèse démontrent que les infections parasitaires sont très fréquentes à Tripoli chez les enfants, indépendamment du statut socio-économique. Une prévalence élevée a été détectée malgré la réalisation de cette étude dans une région urbaine et

sur la collection d'un seul échantillon de selles par enfant, au lieu de trois échantillons consécutifs. Une étude récente en Malaisie chez les écoliers a rapporté une prévalence des infections parasitaires de 98%, dans une zone essentiellement rurale (Al-Delaimy et al., 2014). Les chiffres au Liban sont alarmants en termes d'impact potentiel en santé publique.

Globalement, sur la base de l'utilisation d'outils moléculaires pour la détection de la cryptosporidiose chez les patients hospitalisés (Article 1) et les écoliers (Article 2), la prévalence de *Cryptosporidium* spp. au Liban dans ces deux types de population a été similaire à celle rapportée chez les enfants et les adultes dans un autre pays du Moyen-Orient, le Yémen (10%) (Alyousefi et al., 2013), mais inférieure à celle rapportée chez les enfants dans d'autres pays voisins, comme la Jordanie (19%) (Hijjawi et al., 2010) et l'Egypte (49%) (Helmy et al., 2013) et chez les enfants et les adultes dans d'autres pays en développement, comme l'Afrique du Sud (18%) (Samie et al., 2006), l'Uganda (32%) (Salyer et al., 2012). Les pays développés présentent quant à eux des prévalences généralement inférieures à 2% (ANOFEL, 2010; Fournet et al., 2013; Guyot et al., 2012).

Sur la base des questionnaires remplis pour chaque enfant dans la cohorte des écoliers au Liban, nous avons pu déterminer des facteurs de risque potentiels associés aux infections parasitaires intestinales. Le contact avec des membres de la famille souffrant de troubles gastro-intestinaux a été identifié comme le principal facteur de risque lié à la présence d'infections parasitaires. En ce qui concerne particulièrement la cryptosporidiose, un âge inférieur à 5 ans ainsi que la consommation d'aliments à l'extérieur du domicile étaient identifiés comme les principaux facteurs de risque. D'autre part, un statut socio-économique de bas niveau ainsi que la consommation d'eau non-traitée et de végétaux crus ont été reconnus comme favorisant la giardiose. La cryptosporidiose ainsi que la giardiose ont été corrélées avec la présence de symptômes digestifs chez les enfants.

Ces résultats confirment des données récentes associant l'infection par *Cryptosporidium* spp. à une âge inférieur à 5 ans (ANOFEL, 2010; Kotloff et al., 2013; Striepen, 2013) et l'infection par *Giardia duodenalis* à la consommation d'eau non-traitée et de végétaux crus (Alyousefi et al., 2011; Esrey et al., 1989).

En analysant d'autres parasites, on note de manière intéressante une association statistiquement significative entre la présence de *Blastocystis* sp. et celle de *Dientamoeba fragilis* ($P<0.001$). Cette association a été rapportée auparavant chez des enfants symptomatiques aux Pays-Bas (Maas et al., 2014) et dans une population asymptomatique de bas niveau socio-économique au Brésil (David et al., 2015).

Le génotypage des isolats de *Cryptosporidium* spp. et de *Blastocystis* sp. a permis de mieux comprendre les modalités de la transmission de ces deux parasites au Liban.

Les données de génotypage de *Cryptosporidium* chez les écoliers (Article 2) confirment une prédominance de *C. hominis* également retrouvée dans notre étude chez les patients symptomatiques hospitalisés. Cependant, nous avons identifié des sous-types différents dans chaque population. Le sous-type IdA19, qui a été décrit comme le sous-type prédominant chez les patients hospitalisés libanais, n'a pas été trouvé chez les écoliers. Deux autres sous-types appartenant aux familles Ia et Ib, IaA18R3 (20%) et IbA10G2 (80%), ont été identifiés. Le sous-type IbA10G2 a été fréquemment rapporté dans le monde entier, et représente la principale cause d'épidémies d'origine hydrique par *C. hominis* (Chalmers, 2012). Par contre, IaA18R3 est un sous-type rare récemment signalé en Inde et en Espagne (Fuentes et al., 2014; Sharma et al., 2013). Tous les isolats de *C. parvum* ont été identifiés comme appartenant au sous-type IIaA15G1R1. Ce sous-type zoonotique a été identifié chez les humains et les animaux dans de nombreuses zones géographiques du monde (Ryan et al., 2014). Les familles des sous-types IIc et IId, fréquemment reportées dans la plupart des pays en développement, n'ont pas été décrites au Liban (Adamu et al., 2014; Helmy et al., 2013).

De plus, les résultats de sous-typage des isolats de *Blastocystis* sp. sont cohérents avec ceux de la majorité des études épidémiologiques réalisées à travers le monde avec une prédominance d'isolats de ST3 suivis de ceux de ST1 et ST2. Par contre le ST4, répandu en Europe, n'a pas été trouvé dans notre étude au Liban et serait ainsi beaucoup moins fréquent au Moyen-Orient mais aussi en Afrique, en Amérique et en Asie (El Safadi et al., 2013; Stensvold, 2013).

La prédominance de l'espèce *C. hominis* et des ST1, ST2 et ST3 de *Blastocystis* sp. suggèrent une forte transmission anthroponotique pour ces deux parasites au Liban. Ce résultat est en cohérence avec le fait que le contact avec des membres de la famille souffrant de troubles gastro-intestinaux a été identifié comme le principal facteur de risque lié à la présence d'infections parasitaires (Article 2).

Axe 2 : Etude de la prévalence de *Cryptosporidium* spp. dans les échantillons animaux et l'évaluation du pouvoir zoonotique du parasite

L'importance du réservoir représenté par les animaux de rente ou sauvages pour les différentes espèces de cryptosporidies et l'implication de l'espèce zoonotique *C. parvum* dans plusieurs épidémies conduit à donner une importance particulière à cet aspect. De ce fait, il

nous est paru essentiel de réaliser des études épidémiologiques complémentaires dans des populations d'animaux afin de clarifier les voies de transmission de ce parasite. Du fait du potentiel zoonotique de *Cryptosporidium* spp., confirmé par des données de la littérature récentes (Chalmers and Katzer, 2013; Ryan et al., 2014), une meilleure connaissance de l'épidémiologie moléculaire de ce parasite chez les animaux est un élément clef afin de limiter le risque potentiel de transmission de la cryptosporidiose. Nous avons ainsi cherché à caractériser la prévalence et la variabilité des espèces de *Cryptosporidium* présentes au Liban mais aussi en France chez des animaux d'élevage, de compagnie, en captivité et sauvages.

i) Au Liban :

Avant ce travail de thèse, aucune information n'était disponible concernant la situation de la cryptosporidiose chez les animaux au Liban. De ce fait, une nouvelle étude épidémiologique a été menée dans une région géographique limitée du Nord-Liban, le district d'Akkar (Article 3). Cette étude nous a permis de clarifier la situation de la circulation de *Cryptosporidium* spp. en utilisant la PCR nichée comme méthode d'identification.

De ce fait, nos résultats moléculaires constituent les premières données épidémiologiques sur la cryptosporidiose chez les humains et les bovins au district d'Akkar, région rurale du Nord du Liban, et confirment une prédominance de *C. hominis* dans la population humaine libanaise et de *C. andersoni* chez les bovins. La recherche du parasite dans l'eau de consommation n'a pas été réalisée à cause de difficultés logistiques. La prévalence de la cryptosporidiose chez les sujets immunocompétents (5%) était dans la moyenne de ce qui a été décrit dans le bassin méditerranéen (Abd El Kader et al., 2012; Abu-Alrub et al., 2008; ANOFEL, 2010; Cardona et al., 2011; Rahmouni et al., 2014; Usluca and Aksoy, 2011) mais elle reste en-dessous de ce qui a été décrit dans nos études précédentes réalisées au Liban (Articles 1 et 2). Les enfants sembleraient plus touchés par l'infection que les adultes, comme le rapportent d'autres études notamment en France (ANOFEL, 2010), aux USA (Yoder et al., 2012) et au Royaume Uni (Chalmers et al., 2011).

Par contre, la prévalence de *Cryptosporidium* chez les bovins est similaire à celles décrites dans les pays développés (Coklin et al., 2009; Murakoshi et al., 2012). La prédominance du parasite à l'âge adulte chez les animaux dans la population échantillonnée dans notre étude pourrait expliquer cette faible valeur puisque la prévalence de la cryptosporidiose est maximale chez les veaux et diminue avec l'âge (Fayer et al., 2008; Follet et al., 2011). De même, la prédominance de *C. andersoni* est en cohérence avec celle d'études récentes

rapportant la prédominance de cette espèce chez les bovins adultes (Follet et al., 2011; Rieux et al., 2013c; Zhang et al., 2013).

Le typage nous a permis d'identifier un seul sous-type de *C. hominis* (IdA19) chez les patients hospitalisés à Akkar. C'est le même sous-type rare qui a été décrit dans l'enquête précédente ciblant les patients symptomatiques Libanais et rapporté aussi au Canada (Trotz-Williams et al., 2006) et chez des enfants hospitalisés en Chine (Feng et al., 2012).

Les deux sous-types de *C. parvum*, IIaA15G1R1 et IIaA15G2R1 ont été ainsi mis en évidence chez l'homme et les bovins et représentent des sous-types à transmission zoonotique très répandus dans le monde (Xiao, 2010).

Les bovins seraient donc des réservoirs animaux de contamination potentielle pour l'homme. Ces résultats confirment ainsi ceux des premières études qui nous laissaient présager un mode de transmission anthroponotique (Articles 1 et 2) sans exclure pour autant une transmission zoonotique.

ii) En France :

À ce jour, quelques données relatives aux animaux d'élevages (Follet et al., 2011; Rieux et al., 2013a; Rieux et al., 2013b; Rieux et al., 2013c, 2014) sont disponibles en France. Par contre, même si la France est l'un des pays européen présentant la plus grande population d'animaux de compagnie (Seres, 2011). Très peu d'information étaient disponibles sur l'épidémiologie moléculaire de *Cryptosporidium* spp. chez ces animaux. Il en est de même pour les animaux maintenus en captivité et sauvages. Aussi, il nous a apparu intéressant d'explorer la situation de la cryptosporidiose chez des animaux de compagnie, en captivité et sauvages.

- ***Cryptosporidium* chez les chiens :**

Nous avons pu déterminer dans notre étude (Article 4) une prévalence de 2.6% de *Cryptosporidium* chez les chiens. Cette prévalence est proche de celles décrites dans les populations canines dans d'autres pays, par exemple en Italie (Giangaspero et al., 2006), en Thaïlande (Inpankaew et al., 2007), au Brésil (David et al., 2015; Katagiri and Oliveira-Sequeira, 2008), aux Pays-Bas (Overgaauw et al., 2009), et en Chine (Jian et al., 2014). Cependant, elle est beaucoup plus faible que la prévalence de 26% observée récemment dans une étude menée chez les chiots d'élevage en France (Grellet et al., 2014).

Une telle différence entre ces deux études françaises pourrait s'expliquer notamment par la surpopulation des animaux dans les chenils d'élevage, en considérant que la population de notre étude a été constituée par les chiens de particuliers. De plus, dans les chenils, les chiens étudiés sont majoritairement des chiots (Grellet et al., 2014). Or, comme décrit dans la littérature, ce sont généralement les animaux les plus jeunes qui sont les plus réceptifs et les plus sensibles à l'infection (Derouin et al., 2002). En ce sens, nous avons aussi trouvé dans notre étude que l'infection par *Cryptosporidium* était significativement corrélée avec l'âge et donc, seuls les chiens âgés de moins de 14 semaines étaient touchés par la cryptosporidiose ($P<0.05$). Toutes ces données confirment que la prévalence de la cryptosporidiose canine dépend de l'âge de l'hôte comme suggéré précédemment (Grellet et al., 2014; Hamnes et al., 2007; Lucio-Forster et al., 2010; Mirzaei, 2007; Smith et al., 2014).

Des études récentes décrivent que la plupart des chiens infectés par *Cryptosporidium* spp. sont asymptomatiques (Scorza and Tangtrongsup, 2010). C'est le cas dans notre étude où nous avons trouvé que deux chiens infectés par *C. canis* étaient des porteurs sains. Le troisième chien infecté avait la diarrhée, un signe clinique commun de la cryptosporidiose. Cependant, cet animal était également infecté par un autre parasite pouvant potentiellement causer une diarrhée, *Giardia* spp. (Tysnes et al., 2014). En outre, aucune association avec les symptômes gastro-intestinaux et la présence de *Cryptosporidium* n'a pu être clairement établie dans cette étude mais le nombre de chiens infectés par *C. canis* était faible.

Le génotypage des trois isolats a permis d'identifier *C. canis* qui est connu pour infecter majoritairement les chiens. *C. canis* a pourtant également été retrouvée chez l'homme notamment chez les enfants et les patients immunodéprimés (Lucio-Forster et al., 2010; Xiao et al., 2007). Vu que le taux d'infection par *C. canis* chez les chiens, du moins en France, semble être faible et que la plupart des cas de cryptosporidiose humaine dans le monde sont associés à *C. hominis* et *C. parvum* (Chalmers and Katzer, 2013; Ryan et al., 2014), *C. canis* représente donc très probablement un risque zoonotique faible pour la population. Cependant, *C. canis* pourrait être plus dangereux chez les personnes immunodéprimées ayant des contacts avec des chiens infectés (Xiao, 2009).

- ***Cryptosporidium* chez les animaux en captivité dans deux parcs zoologiques français :**

Dans la même optique, nous avons poursuivi nos études épidémiologiques en ciblant deux parcs zoologiques français en l'occurrence les parcs zoologiques de la Palmyre et de Lille.

Ainsi, plus d'une centaine d'espèces animales différentes ont été ainsi analysées pour la présence de *Cryptosporidium* spp. et une prévalence de *Cryptosporidium* presque négligeable (1%) a été rapportée chez un grand nombre d'animaux hébergés dans ces deux parcs..

Parmi les 207 échantillons de selles collectés au zoo de la Palmyre, deux cas positifs de *Cryptosporidium* ont été identifiés chez des oiseaux. Le génotypage de ces isolats a permis l'identification de deux espèces : *C. galli* et *C. andersoni*. *C. galli* est une espèce spécifique des oiseaux (Ryan et al., 2003). Par contre, il est intéressant de remarquer que *C. andersoni* est une espèce qui a été isolée auparavant chez les bovins et chez l'homme mais jamais chez les oiseaux (Chalmers and Katzer, 2013; Ryan et al., 2014). La cohabitation des oiseaux de l'espèce *Struthio camelus* dans le même enclot avec les bovins a pu favoriser une transmission croisée par *C. andersoni* même si cette espèce de *Cryptosporidium* n'a pas été identifiée dans notre étude chez ces mêmes bovins.

Parmi les 98 échantillons de selles collectés au zoo de Lille, *C. tyzzeri*, espèce répandue chez les souris, a été la seule espèce identifiée chez des serpents de genre *Lampropeltis getula*. Nous spéculons qu'une transmission croisée par *C. tyzzeri* a pu être favorisée par le fait que les serpents dans ce parc zoologique sont nourris avec des rongeurs. Nous n'avons pas pu établir si les parasites des espèces *C. andersoni* et *C. tyzzeri* pouvaient développer une infection chez les oiseaux et les serpents, ou si ces animaux n'étaient que des porteurs sains du parasite. On sait aujourd'hui que la spécificité d'hôte chez *Cryptosporidium* est plus ou moins stricte et que des isolats provenant de différents animaux pouvaient se transmettre d'une espèce hôte à une autre, même si les cas de transmission entre différents groupes de vertébrés sont rares (Xiao et al., 2004) . Cependant, *C. meleagridis*, une espèce généralement trouvée chez les oiseaux est capable d'infecter l'homme (Chalmers and Katzer, 2013; Ryan et al., 2014).

La faible prévalence de *Cryptosporidium* dans cette étude pourrait s'expliquer par le fait que l'environnement des animaux hébergés dans ces parcs est très propre et soigné en réponse à des attentes très strictes en ce qui concerne l'hygiène. Ces zoos sont reconnus pour ses améliorations constantes concernant la qualité de vie des animaux. De plus, le zoo de la Palmyre est membre actif de nombreuses associations internationales reconnues pour la conservation des espèces menacées telles que l 'Association pour la Préservation des Primates d'Afrique de l'Ouest (WAPCA) et l'Association Européenne pour l'Etude et la Conservation des Lémuriens (AEECL). Ces associations demandent aux parcs zoologiques une conformité aux normes des programmes d'élevage en environnement contrôlé (El Safadi, 2014).

Bien que la transmission zoonotique du parasite entre les animaux soit possible, nous avons pensé que l'eau potentiellement souillée dans l'environnement des parcs, pouvait jouer un rôle important dans la transmission du parasite, particulièrement dans le cas du parc zoologique de Lille ou certains enclos sont à proximité de la Deûle. Cependant, dans ces environnements contrôlés la probabilité pour que l'eau en contact avec les différentes espèces animales soit de mauvaise qualité semble relativement faible.

Néanmoins, la récolte des échantillons a été effectuée pendant quelques jours du mois d'avril dans le zoo de la Palmyre et du mois de juin dans le zoo de Lille. Avec le but d'étudier l'effet de la saisonnalité, des nouvelles recherches de *Cryptosporidium* spp. en réalisant des collectes de selles à des périodes de l'année différentes devraient être envisagées.

- ***Cryptosporidium* chez les poissons :**

Afin d'améliorer la sécurité alimentaire des produits issus de la filière pêche et de combler une manque de données sur la prévalence des parasites de poisson présentant un impact en santé humaine et/ou un intérêt scientifique majeur en France, le laboratoire BDPEE a été impliqué dans la coordination du projet Fish-Parasites (ANR-ALIA 2010) dans lequel j'ai eu l'occasion de participer en tant que doctorant.

Je me suis impliquée particulièrement dans l'identification des protistes intestinaux appartenant au genre *Cryptosporidium*. En effet, en dépit d'études récentes rapportant l'identification moléculaire de *Cryptosporidium* (Koinari et al., 2013; Morine et al., 2012) dans les poissons, aucun recherche n'avait été conduite en France à ce sujet avant ce travail.

Ce parasite a été recherché dans les muqueuses intestinales et stomacales de 42 poissons d'eau douce provenant du lac Léman (Article 5). L'extraction d'ADN standardisée en microplaques, ainsi que la PCR nichée ciblant le locus de l'ARNr 18S ont été réalisées afin de rendre possible la détection de *Cryptosporidium* spp chez les poissons. La présence de *Cryptosporidium* spp a été mise en évidence chez 37% des poissons. L'espèce *C. parvum* a été détectée majoritairement chez les poissons d'eau douce puisqu'elle a été identifiée dans près de 86% des poissons. La prévalence de *C. molnari* était de 14%. Concernant la localisation des deux espèces du parasite dans les muqueuses stomacales ou intestinales, nos observations confortent ce qui avait été déjà rapporté, à savoir la localisation fréquente de *C. molnari* dans l'estomac (Alvarez-Pellitero and Sitja-Bobadilla, 2002) et celle de *C. parvum* plus fréquemment dans l'intestin (Reid et al., 2010). L'examen microscopique des tissus de poissons infectés par *C. parvum* a révélé la présence de différents stades évolutifs du parasite

au niveau de la bordure apicale de l'épithélium gastrique et intestinal, ce qui plaiderait plutôt en faveur d'une réelle infection du poisson plutôt que d'un simple portage (Article 5, Figure 2).

Afin de déterminer si les filets de poissons avaient été contaminés par des parasites du genre *Cryptosporidium* en provenance du tube digestif, les filets de 100 perches (*Perca fluviatilis*) ont été analysés et nous n'avons confirmé la présence *C. molnari* que dans les filets d'un seul individu.

Afin de mieux cerner les conditions de la transmission de *C. parvum* dans les poissons du lac Léman, un génotypage a été réalisé au locus gp60 (Alves et al., 2003). Les sous-types IIaA15G2R1, IIaA16G2R1 et IIaA17G2R1 retrouvés sont des génotypes de *C. parvum* provenant de bovins. Le sous-type IIaA15G2R1 que nous avons aussi trouvé chez les bovins dans le district d'Akkar (Article 3) est considéré comme le sous-type le plus répandu en Europe (Xiao, 2010), ce qui atteste d'une forte activité d'élevage en périphérie du lac.

D'autre part, notre étude a permis d'identifier cinq nouvelles espèces de poissons comme de nouveaux hôtes pour *Cryptosporidium* : l'omble chevalier (*Salvelinus alpinus*), le brochet (*Esox lucius*), le corégone européenne (*Coregonus lavaretus*), la perche européenne (*Perca fluviatilis*) et le gardon (*Rutilus rutilus*).

Toutes les données moléculaires obtenues dans cet axe de recherche montrent que les animaux des parcs zoologiques et les chiens domestiques en France sont peu infectés par *Cryptosporidium* et semblent être des réservoirs potentiels mais négligeables pour la transmission de ce parasite en France.
Par contre dans notre étude chez les poissons, deux espèces de *Cryptosporidium* ont été trouvées: *C. molnari* qui a été décrit précédemment chez des poissons marins (Alvarez-Pellitero and Sitja-Bobadilla, 2002) mais qui a été observé pour la première fois dans notre étude chez les poissons d'eau douce et *C. parvum*, trouvé avec une forte prévalence, déjà identifié chez les poissons (Reid et al., 2010) mais considéré essentiellement comme une espèce infectant les mammifères (Lindsay et al., 2000; Ryan and Power, 2012). De plus, les analyses histologiques que nous avons réalisées nous ont permis de montrer que la présence de *C. parvum* chez les poissons semblerait plus correspondre à une infection qu'à un portage. Le maintien du cycle de *C. parvum* chez les poissons pourrait ainsi représenter un indicateur de pollution fécale du milieu lacustre.

Ces observations ont un impact potentiel en santé publique car *C. parvum* est une espèce zoonotique et que la dispersion de ce parasite par les poissons serait facilitée par l'habitat aquatique de l'hôte (Wilkes et al., 2013). De plus, en termes d'infectiosité, il est connu qu'un seul oocyste de *C. parvum* est capable d'induire une infection chronique (Benamrouz et al., 2012b). Nous avons également identifié la présence de *Cryptosporidium* dans les filets. Cette pêche locale est consommée directement par les pécheurs ou vendue en poissonnerie ou aux nombreux restaurants situés sur les bords du lac, qui proposent parfois des recettes à base de poissons crus. A la vue de nos données, il est donc nécessaire d'informer les consommateurs sur les risques liés à la consommation de poissons crus ou mal cuits dans cette région.

Axe 3 : Pathogénicité

Les objectifs précédents de ce travail de thèse permettaient d'établir que *Cryptosporidium* est un parasite ubiquiste et peu spécifique, capable d'infecter de nombreuses hôtes. Le potentiel zoonotique du parasite est cependant variable et dépend des diverses espèces et sous-types aujourd'hui décrits. Par ailleurs, des cryptosporidies issues d'espèces non mammifères comme les poissons, peuvent aussi se retrouver dans l'environnement.

En conséquence, tout oocyste de cryptosporidie devrait être considéré comme potentiellement dangereux pour les humains**.** Dans ce contexte, toutes les sources de contamination d'origine animale (domestique et sauvage) et humaine doivent être prises en considération, ainsi que leur diffusion et persistance dans l'environnement (Derouin et al., 2002).

De ce fait, le troisième axe de ma thèse a comme objectif l'étude de la pathogénicité de ce parasite. Il a été décrit précédemment que chez l'homme ou l'animal la sévérité de la cryptosporidiose était variable en fonction de leur susceptibilité ainsi que de la pathogénicité intrinsèque des isolats (Okhuysen and Chappell, 2002). De plus, l'ensemble de données expérimentales chez l'animal (Benamrouz et al., 2012a; Benamrouz et al., 2012b; Certad et al., 2012; Certad et al., 2010a; Certad et al., 2010b; Certad et al., 2007) et clinico-épidémiologiques chez l'homme (Patel et al., 2008; Shebl et al., 2012; Sulzyc-Bielicka et al., 2012; Sulzyc-Bielicka et al., 2007; Yeguez et al., 2003) montrant une association entre la pathologie cancéreuse et le parasitisme par *Cryptosporidium* suggère fortement que le spectre de pathogénicité de *Cryptosporidium*, au moins de l'espèce *C. parvum*, comporte vraisemblablement un pouvoir carcinogène.

- Y a-t-il une association entre la cryptosporidiose et les néoplasies digestives chez l'homme ?

Pour répondre à cette question nous avons voulu réaliser une étude visant à rechercher la présence du parasite dans des biopsies coliques et gastriques inclues en paraffine de patients atteints ou non de cancer de la région Nord-Liban (Article 6) où comme décrit précédemment nous avons trouvé une forte prévalence de cryptosporidiose dans différentes cohortes de la population (Articles 1, 2 et 3).

De manière intéressante, une forte prévalence de la cryptosporidiose (16.3%) a été détectée chez des patients libanais adultes atteints de néoplasies digestives, de diagnostic récent et avant tout traitement. Cette prévalence est encore plus élevée chez les patients atteints de cancer colorectal (21.1%). La population témoin, constituée de patients avec des troubles digestifs mais non porteurs de néoplasies a montré une prévalence plus faible (7%), similaire à celle de la population générale au Liban (Article 3). L'étude histologique a permis la confirmation de la présence de différents stades évolutifs du parasite au niveau de lésions néoplasiques (Article 6, Figure 1).

Dans le but d'exclure la présence d'autres pathogènes oncogènes liés à l'induction du cancer du côlon (Fiorina et al., 2014), une recherche pilote a été réalisée afin de cibler le virus d'Epstein Barr (EBV) dans les biopsies. Malgré le faible nombre d'échantillons analysés, nous avons trouvé une distribution homogène de l'EBV dans les deux groupes infectés ou non par *Cryptosporidium* ainsi que dans les deux populations cible et témoin. Ces résultats sont cohérents avec le fait que l'EBV peut infecter la population humaine très tôt en persistant tout au long de la vie (Parkin, 2011). Des études complémentaires doivent être réalisées sur un nombre plus important d'échantillons afin de clarifier cet aspect.

L'étude de la diversité génétique des isolats a montré une prédominance de *C. hominis* dans les biopsies des deux populations cible et témoin. La prédominance de *C. hominis* a aussi été retrouvée dans nos différentes études réalisées au Liban (Articles 1, 2 et 3). Cependant, l'association entre le cancer digestif et la présence de *C. hominis* est rapportée pour la première fois dans notre travail de thèse. Cette espèce a été testée auparavant dans un modèle expérimental mais les souris SCID traitées ou non avec la Dex ont été réfractaires à l'infection due à la spécificité d'hôte de *C. hominis* considérée plutôt comme une espèce inféodée à l'homme, même si elle peut infecter expérimentalement d'autres mammifères (Certad et al., 2010a).

Des données ont déjà été rapportées concernant l'association entre la présence de *Cryptosporidium* et le développement d'un cancer digestif chez l'homme. Une association

entre un adénocarcinome du colon diagnostiqué sur une biopsie chez un patient espagnol de 64 ans, et la présence d'oocystes de *Cryptosporidium* a été décrite (Izquierdo et al., 1988). Plus récemment, une forte prévalence de *Cryptosporidium* a été montrée chez des patients atteints de cancer colorectal en Pologne (Sulzyc-Bielicka et al., 2012; Sulzyc-Bielicka et al., 2007). En outre, le cancer colorectal a été identifié comme une des "non-AIDS defining malignancies" avec une incidence de plus en plus élevée chez les patients VIH+ par rapport à la population générale (Patel et al., 2008; Yeguez et al., 2003). Une analyse épidémiologique rétrospective réalisée aux Etats Unis a permis de conclure que la cryptosporidiose, comme l'herpès mucocutané, augmentait significativement le risque de cancer colorectal chez une population de personnes atteintes du SIDA (Shebl et al., 2012). De plus, *C. parvum* semble être associé à des diarrhées chroniques associées au cancer du foie chez des enfants atteints du syndrome « X-linked hyper-IgM » (Hayward et al., 1997).

Toutefois, dans tous ces rapports il est difficile de savoir si *Cryptosporidium* se comporte comme un facteur de carcinogenèse ou simplement comme un agent opportuniste dont le développement a été renforcé par l'immunosuppression de l'hôte.

Cependant, ce travail de thèse fournit les premières données épidémiologiques mettant en évidence une association directe entre la présence de *Cryptosporidium* au niveau du tissu épithélial et le cancer digestif. Ceci constitue un argument fort en faveur de l'hypothèse de l'association de *Cryptosporidium* au développement de cancers digestifs chez l'homme.

- **Cancer colique induit par *C. parvum* chez le modèle murin: Quel sont les hypothèses sur les mécanismes de la carcinogénèse ?**

Dans un autre volet de mon travail de thèse, je me suis intéressé aux voies métaboliques potentiellement impliquées dans le processus d'induction des lésions néoplasiques par *C. parvum* (IOWA) au niveau de la région iléocæcale. Ainsi, quatre marqueurs connus comme étant impliqués dans les principales voies altérées lors du développement de cancers colorectaux (Sancho et al., 2004) ont été sélectionnés: APC et Bêta-caténine qui appartiennent à la voie de signalisation Wnt ainsi que P53 et K-ras (Article 7).

Des études immunohistochimiques ont été réalisées et ont permis de montrer une localisation anormale de l'APC, de la Bêta-caténine et de la P53 dans les cellules épithéliales néoplasiques. En effet, le marquage de l'APC dans les cellules néoplasiques diminue (Article 7, Figure 1). D'autre part la Bêta-caténine, normalement localisée au niveau de la membrane cellulaire et la P53, communément localisée dans le noyau, s'accumulent dans le cytoplasme (Article 7, Figure 2). Dans le but de confirmer cette localisation cytoplasmique de la Bêta-

caténine, nous avons réalisé une analyse par immunofluorescence sur des coupes histologiques de la région iléocæcale de souris infectées par *C. parvum* et atteintes d'un adénocarcinome invasif après 90 jours post-infection (P.I.). Une accumulation de la Bêta-caténine a été observée principalement au niveau basolateral de la cellule en zone juxtamembranaire et l'absence de Bêta-caténine dans le noyau a été confirmée (Article 7, Figure 2). L'analyse par Western Blot réalisée tout d'abord, sur des extraits totaux de cellules a montré une augmentation de l'expression de la Bêta-caténine. La même analyse réalisée après fractionnement des cellules tumorales chez les souris infectées par *C. parvum* a permis de mettre en évidence des traces de Bêta-caténine nucléaire (fraction F3) mais dans les mêmes proportions que celles observées chez les animaux contrôles. Néanmoins, une augmentation de la Béta-caténine a été mise en évidence dans la fraction F2 correspondant à la membrane et aux organelles des tumeurs iléocæcale de souris infectées par *C. parvum* et porteuses de la néoplasie (Article 8, Figure 2). Une analyse de l'interaction entre des cellules hôtes et le parasite par microscopie électronique a permis l'observation des espacements intercellulaires avec apparition de prolongements cytoplasmiques latéraux aussi bien dans la région iléocæcale qu'antero-pylorique des souris infectées avec *C. parvum*, alors que l'observation des régions antero-pyloriques des souris infectées avec *C. muris* ne montre pas ce type de modification. Enfin, un séquençage haut-débit de *loci* cibles (Bêta-caténine, APC et K-ras) décrits dans la littérature comme étant les plus fréquemment mutés dans les cas de cancer colorectaux chez la souris (Kuraguchi et al., 2000; Miyaki et al., 1999) n'a pas permis de mettre en évidence de mutations.

L'ensemble de ses observations semble donc traduire que *C. parvum* est capable d'induire un adénocarcinome iléocæcale en modifiant le cytosquelette de la cellule hôte, probablement via l'altération de la voie Wnt qui est connue pour son implication dans le processus de polymérisation de l'actine et dans le réarrangement du cytosquelette pendant le processus de cancérisation (Giles et al., 2003). Particulièrement, ceci est intéressant étant donné qu'il a été décrit que l'infection par *Cryptosporidium* au moment de l'invasion du parasite dans la cellule épithéliale régule la réorganisation de l'actine et induit des changements au niveau du cytosquelette de la cellule hôte à travers des voies de signalisation telles que la voie de la phosphatidyinositol-3-kinase (PI3K) (O'Hara & Chen 2011).

En conclusion, cette étude a permis de montrer que *C. parvum* indépendamment de la souche est donc capable de moduler les activités du cytosquelette ainsi que des voies de signalisations de la cellule hôte. Cependant, il est encore difficile de conclure l'origine spécifique de la transformation cellulaire induite par *Cryptosporidium*. Pourtant il est possible que la

combinaison multifactorielle de plusieurs processus soit responsable de la transformation des cellules épithéliales coliques.

VIII. Conclusions et Perspectives

Ce travail de thèse a été réalisé dans le cadre d'un projet collaboratif entre le Liban et la France. Il avait pour but d'étudier l'épidémiologie moléculaire de la cryptosporidiose et l'association entre la pathologie cancéreuse et l'infection par *Cryptosporidium* chez l'homme mais également d'explorer les mécanismes de cancérogénèse induits par *C. parvum* chez les souris SCID. Les conclusions de ce travail sont les suivantes :

1. Les premières données épidémiologiques concernant la cryptosporidiose dans des populations de patients libanais symptomatiques et asymptomatiques et chez les bovins au Liban ont été obtenues.
2. Une forte prévalence de *Cryptosporidium* spp a été mise en évidence aussi bien dans la population symptomatique que dans la population générale. Ces prévalences suggèrent que ce protiste est un véritable problème de santé publique dans cette région.
3. Les données de génotypage de *Cryptosporidium* au Liban montrent une prédominance de *C. hominis* dans toutes les populations humaines libanaises ciblées. Cependant une autre espèce, *C. parvum*, a également été rapportée aussi bien chez l'homme que chez les bovins.
4. L'analyse moléculaire réalisée à l'aide du marqueur gp60 nous a permis d'identifier les sous-types de *C. hominis* et *C. parvum* circulants afin de clarifier le type de transmission de l'infection. Ces résultats suggèrent une prédominance de la transmission anthroponotique sans exclure pour autant la voie zoonotique.
5. Nos données ont également mis l'accent sur le fait que globalement les infections parasitaires intestinales sont très fréquentes à Tripoli chez les enfants, indépendamment de leur statut socio-économique. Un certain nombre de facteurs de risque potentiels associés aux parasites intestinaux ont été identifiés. Le plus important est dans notre étude, le contact avec des membres de la famille souffrant de troubles gastro-intestinaux. En ce qui concerne plus particulièrement *Cryptosporidium,* un âge inférieur à 5 ans et la consommation d'aliments hors du domicile sont les principaux facteurs mis en évidence.
6. Ce travail a pu confirmer que *Cryptosporidium* est un parasite ubiquiste et peu spécifique, capable d'infecter de nombreux hôtes. Le potentiel zoonotique du parasite est cependant variable et dépend des diverses espèces et sous-types.
7. *Cryptosporidium* semble être largement distribué dans les écosystèmes puisqu'une prévalence élevée du parasite a été trouvée chez les poissons d'eau douce. De plus, dans le cadre de nos travaux, 5 espèces de poissons ont été identifiées comme étant de nouveaux hôtes pour *Cryptosporidium*. L'ensemble des résultats obtenus pour

Cryptosporidium laisse à penser que les poissons, et plus particulièrement ceux d'eau douce, pourraient participer à la transmission de *ce parasite* et de ce fait, des règles de prévention doivent être mises en place.

8. Une évaluation de la prévalence de la cryptosporidiose chez des patients libanais, porteurs ou non de néoplasies digestives, nous a permis de montrer une association significative entre la présence de *Cryptosporidium* dans les tissus et la présence des néoplasies digestives, particulièrement coliques. Deux espèces de *Cryptosporidium* ont été associées aux lésions néoplasiques : *C. parvum* et *C. hominis*. Pour la première fois, *C. hominis,* espèce inféodée à l'homme, a également été associée à ce processus de cancérogénèse.
9. L'exploration des voies de signalisation cellulaires impliquées dans le développement des néoplasies digestives chez les souris SCID-D infectés par *C. parvum* a été initiée. L'étude des altérations génomiques ou protéiques associées à l'infection parasitaire nous a conduit à suggérer que ce protiste est capable d'altérer le cytosquelette, mais également d'impliquer la voie Wnt lors du processus de cancérogénèse. Plusieurs marqueurs comme la bêta-caténine, l'Apc et la p53 ont été étudiés. Leur détection par immunofluorescence et western blotting a permis de mettre en évidence une localisation anormale au niveau des cellules néoplasiques. A ce jour, les processus et les molécules par lesquels *C. parvum* agirait sur la cellule hôte ne sont pas clairs. Plusieurs hypothèses ont été proposées mais elles doivent être confirmées car il s'agit très probablement d'un processus multifactoriel.
10. Tous ces éléments devraient accentuer l'intérêt porté à ce parasite auquel un grand nombre de personnes et d'animaux sont exposés. Particulièrement, la découverte d'une association entre l'infection par *Cryptosporidium* et le développement du cancer digestif chez l'homme semble d'un intérêt majeur pour les recherches visant à comprendre le processus de cancérogenèse colorectale.

Afin de compléter ce travail, nous pourrions envisager, à court et moyen terme, différentes voies d'exploration :

- Pour mieux comprendre la transmission de la cryptosporidiose au Liban, une recherche et une quantification des oocystes de *Cryptosporidium* dans l'alimentation et dans l'eau sera initiée. Ainsi, un projet financé par le programme Hubert Curien CEDRE 2015 a été mis en place dans l'équipe BDPEE afin d'évaluer le risque de transmission de *Blastocystis* sp. et *Cryptosporidium* spp. à l'homme par la filière poulet de chair et son impact en santé publique. L'étude de cette population ainsi que la détection des oocystes de *Cryptosporidium* dans des échantillons environnementaux aidera à mieux clarifier le risque de transmission de ces agents à l'homme et la mise en place d'actions de surveillance de ces parasites.
- Concernant l'association *Cryptosporidium*/cancer, l'exploration d'éventuelles altérations au niveau d'autres gènes impliqués dans le processus de tumorogenèse colique comme la PI3K doit être envisagée ainsi que la recherche d'altérations transcriptomiques et épigénétiques des entérocytes dans le modèle animal. Cette approche nous permettra d'identifier la signature moléculaire des néoplasies induites par *Cryptosporidium* et ainsi la rechercher dans les tumeurs coliques chez l'homme.
- L' identification de gènes potentiellement impliqués dans la transformation de la cellule hôte par une analyse *in silico* des génomes de *Cryptosporidium* déjà séquencés (Abrahamsen et al., 2004; Xu et al., 2004) et disponibles dans la base de données (CryptoDB) (Puiu et al., 2004) est en cours.
- L'étude de l'influence que pourrait avoir la flore intestinale sur l'induction des néoplasies par *C. parvum* a été initiée par une analyse comparative du microbiote intestinal de souris SCID-Dex infectées par *C. parvum* et de souris infectées par *C. muris*. Ceci nous permettra d'identifier une éventuelle dysbiose associée spécifiquement à l'infection par *C. parvum*.
- Afin de confirmer l'implication directe du parasite dans l'induction de la transformation maligne des cellules hôtes chez l'homme, les prévalences de l'infection par *Cryptosporidium* chez des patients présentant un cancer digestif diagnostiqué récemment et cela avant tout traitement, seront déterminées dans d'autres zones géographique.

IX. *Annexes*

1. Travaux présentés aux congrès scientifiques

11ème Congrès National de la SFM : 23-25 Mars 2015, Institut Pasteur, Paris, France.

9th Dubai Food Safety Conference: 9-11 Novembre 2014, Dubaï, Émirats arabes unis.

New insights into the molecular epidemiology and transmission dynamics of *Cryptosporidium* spp. and *Blastocystis* sp. in North Lebanon

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Abstract

Cryptosporidium sp. and *Blastocystis* sp. are two protists with worldwide distribution that infect the gastrointestinal tract of several vertebrates including humans. Human to human, zoonotic, foodborne and waterborne are reported as the main transmission routes of these two parasites. The prevalence of *Blastocystis* sp., the most commonly isolated human intestinal protozoan in children and adults can reach until 100% in some populations. *Cryptosporidium* is less prevalent but it has been recognized as the predominant cause of waterborne and foodborne outbreaks. The epidemiological situation of these infections is not well known in Lebanon, a developing country with a population often affected by other intestinal parasitic infections. Therefore, we aimed to study the genetic diversity and routes of transmission of these two parasites in the Akkar district of the North of Lebanon. One hundred human fecal

samples were obtained from both symptomatic and asymptomatic patients in two hospitals of this region (Al-Youssef Hospital and Rahal Hospital). In addition, 152 fecal samples from cattle (Holstein breed) were collected randomly from 33 farms or barns located in 20 villages. Environmental samples like water (one liter from each sampling area) and raw vegetables (25g of each kind) were also collected. After DNA extraction, detection and identification of *Cryptosporidium* sp. and *Blastocystis* sp. was performed independently by PCR and sequencing using specific primers targeting the 18S rRNA gene. Overall, after molecular analysis of human fecal samples, *Cryptosporidium* sp. and *Blastocystis* sp. were found in 5% and 30% of samples, respectively. In addition, 8% and 80% of the cattle fecal samples were found to be positive for *Cryptosporidium* sp. and *Blastocystis* sp., respectively. Furthermore, *Blastocystis* water contamination was revealed in 69 out of 97 water samples. *Cryptosporidium* was not found in water but this negative result could be attributed to the low volume of water samples intended to *Cryptosporidium* diagnosis. The detection of parasites in raw vegetable samples is still in progress. *C. hominis* (80%) and *C. andersoni* (50%) were found to be the predominant *Cryptosporidium* species in humans and cattle, respectively. However, *C. parvum*, a zoonotic species, was present in less proportion in both populations. In relation to *Blastocystis*, ST3 was the most common ST found in human (67%) and water samples (50%), followed by ST1, ST2 and ST4. However, ST10 (45%) and ST14 (45%) were the most predominant STs found in cattle. Only one ST10 isolate was identified in water samples. In parallel, subtype analysis of human isolates of *C. hominis* and human and cattle isolates of *C. parvum* at the GP60 locus, allowed the identification of the subtype IdA19 of *C. hominis,* and the subtypes IIaA15G1R1 and IIaA15G2R1 of *C. parvum*. The predominance of different species of *Cryptosporidium* in the two populations (*C. hominis* in patients and *C. andersoni* in cattle), suggests an anthroponotic rather than a zoonotic transmission of this parasite in the area of study, even though zoonotic transmission from dairy cattle may eventually occur, as attested by the presence of common subtypes of *C. parvum* for both. Similarly, current findings showing different kind of subtypes of *Blastocystis* infecting both human and animals highlight the predominance of an anthroponotic transmission. Besides, the waterborne transmission of *Blastocystis* seems to be important. Our results reveal that these infections are highly prevalent in the studied area of Lebanon. These findings support a need of a control program to prevent the circulation of these parasites.

New insights into the molecular epidemiology and transmission dynamics of *Cryptosporidium* spp. and *Blastocystis* spp. in North Lebanon

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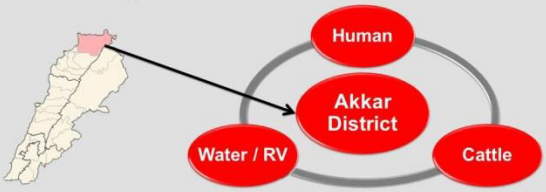
Introduction

Eukaryotic pathogens including protozoa are responsible for emergent diseases and currently constitute major public health issues. However, the diseases they cause are usually neglected or considered "rare" by health authorities. Two of these pathogens, *Cryptosporidium* spp. and *Blastocystis* spp. are protists with worldwide distribution that infect the gastrointestinal tract of several vertebrates including humans. Human to human, zoonotic, foodborne and waterborne are reported as the main transmission routes of these two parasites. The prevalence of *Blastocystis* spp., the most commonly isolated human intestinal protozoan in children and adults can reach until 100% in some populations. *Cryptosporidium* is less prevalent but it has been recognized as the predominant cause of waterborne and foodborne outbreaks. The epidemiological situation of these infections is not well known in Lebanon, a developing country with a population often affected by other intestinal parasitic infections. Therefore, we aimed to study the genetic diversity and routes of transmission of these two parasites in the Akkar district, a rural area of the North of Lebanon.

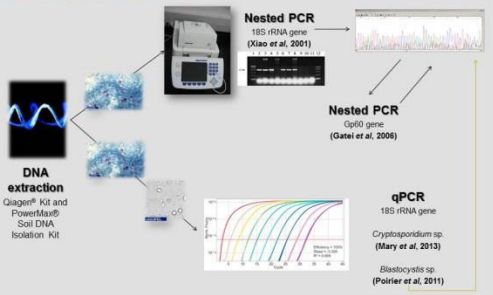


Materials and Methods

Sample collection:

One hundred human fecal samples were obtained from both symptomatic and asymptomatic patients in two hospitals of the Akkar district (Al-Youssef Hospital and Rahal Hospital). In addition, 152 fecal samples from cattle (*Holstein breed*) were collected randomly from 33 farms or barns located in 20 villages. Environmental samples like water (one liter from each sampling area, N=97) and raw vegetables (RV) (25g of each kind, N=35) were also collected. After DNA extraction, detection and identification of *Cryptosporidium* sp. and *Blastocystis* sp. was performed independently by PCR and sequencing using specific primers targeting the 18S rRNA gene.

Detection and molecular characterization of *Cryptosporidium* and *Blastocystis* isolates:



Results

Table 1. Prevalence of *Cryptosporidium* and *Blastocystis* (18S rRNA gene)

	Human (stools)	Cattle (stools)	Water	Raw Vegetables
Prevalence of *Cryptosporidium* spp. (%)	5/100 (5%)	12/152 (8%)	0/30 (0%)	6/35 (17%)
Prevalence of *Blastocystis* spp. (%)	30/100 (30%)	122/152 (80%)	69/97 (68%)	In progress

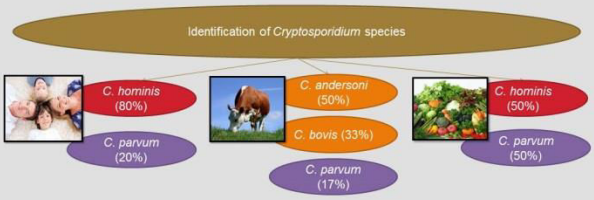


Table 2. Identification of *Blastocystis* subtypes

Samples	ST1	ST2	ST3	ST4	ST5	ST7	ST10	ST14	Mixed infection
Water	13 (20%)	10 (16%)	32 (50%)	8 (13%)	-	-	1 (1%)	-	5
Human (stools)	5 (17%)	3 (10%)	20 (67%)	2 (6%)	-	-	-	-	-
Cattle (stools)	4 (4%)	-	-	-	5 (6%)	1 (1%)	41 (44.5%)	41 (44.5%)	29

- **The subtype analysis** of human isolates of *C. hominis* and human and cattle isolates of *C. parvum* at the GP60 locus, allowed the identification of the subtype IdA19 of *C. hominis,* and the subtypes IIaA15G1R1 and IIaA15G2R1 of *C. parvum*.

Discussion and Conclusion

- The prevalence of *Cryptosporidium* and *Blastocystis* in both humans and animals varies between and within countries in the world.
- The predominance of different species of *Cryptosporidium* in the two populations (*C. hominis* in patients and *C. andersoni* in cattle), suggests an anthroponotic rather than a zoonotic transmission of this parasite in the area of study, even though zoonotic transmission from dairy cattle may eventually occur, as attested by the presence of common subtypes of *C. parvum* for both.
- The current findings showing different kind of subtypes of *Blastocystis* infecting both human and animals highlight the predominance of an anthroponotic transmission of this parasite.
- A high prevalence of *Cryptosporidium* in raw vegetables was found (17%). *Cryptosporidium* was not found in water but this negative result could be attributed to the low volume of water samples intended to *Cryptosporidium* diagnosis.
- The waterborne transmission of *Blastocystis* and the foodborne transmission of *Cryptosporidium* via RV seems to play a role in the epidemiology of these infections.
- These findings support a need of a control program to prevent the circulation of these parasites.

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Identification of *Cryptosporidium* species in fresh water and marine fish in France

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Cryptosporidium is a protist parasite affecting a wide range of hosts worldwide. The parasite is mainly transmitted *via* the fecal-oral route or *via* contaminated water or food and it may cause enteric infection in both humans and animals. The disease results in severe diarrhea and can be life threatening, particularly in very young and immunosuppressed individuals. More than 20 *Cryptosporidium* species have been described infecting different vertebrates. Particularly, *Cryptosporidium* has been genetically characterized in 19 species of fish. Nevertheless, only few data are currently available regarding the characterization of *Cryptosporidium* species and genotypes in natural aquatic environments, and in particular in edible fish. The main objective of our study was to determine the prevalence of *Cryptosporidium* sp. in edible fish products in France (fresh water and marine fish). In total, 1512 fish were collected during fishing sea campaigns along the French coasts, and from fisheries suppliers at Thonon-les-Bains at the border of the Geneva lake (Lac Léman), between 2011 and 2013. Stomachal and intestinal epithelial cells were scrapped off and placed into tubes containing the fixative reagent, RCL2. After DNA extraction, detection and

identification of *Cryptosporidium* spp. was performed by PCR-sequencing using degenerated primers targeting the 18S rRNA gene. Subtype identification of *C. parvum* was subsequently done by amplification and sequencing of the GP60 gene. Histological examination of tissues was also conducted. In the Geneva lake the presence of *Cryptosporidium* was detected in 15 fish, leading to a global prevalence of 33% distributed as follows: 86% (13) of the fish were infected by *C. parvum*, 7% (1) by *C. molnari* and 7% (1) had mixed infection by *C. molnari* and *C. parvum*. When the analysis of the locus GP60 was done for *C. parvum*-positive fish, the subtypes IIaA15G2R1 and IIaA13G2R1 were found. In marine fish, molecular analysis allowed the identification of *Cryptosporidium* spp in 28 fish samples, which represents a prevalence of 2% distributed as follows: 25% (7) of the fish were positive for *C. parvum* and 75% (21) for *C. molnari*. Overall, 5 species of fresh water fish and 11 species of marine fish were identified as new hosts for *Cryptosporidium*. Microscopical examination of *C. parvum*-infected fish allowed the identification of different stages of parasites in the apical border of the gastric and intestinal mucosa which suggests that *C. parvum* is truly infecting fish rather than being passively carried. The transmission and dispersion of fish parasites are facilitated by the aquatic habitat of the host that could potentially release fully sporulated oocysts contributing to a perpetuation of the *Cryptosporidium* transmission. In addition, the detection of *C. parvum* in fish is of significance to public health as these species represent the most common source of human infection. Finally, fish may be a good sentinel for the detection of water contamination by sewage or agricultural run-off. This work was supported by the French National Research Agency (grant No. ANR-10-ALIA-004) and the regional pole of competitiveness AQUIMER.

Identification of *Cryptosporidium* species in fresh water and marine fish in France

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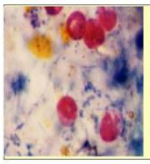
Protozoan and metazoan parasites frequently infest edible fishes worldwide. Some of them are both fish pathogens and recognized agents of important zoonoses with high public health impact



Cryptosporidium **is a protist parasite affecting a wide range of hosts worldwide. The parasite is mainly transmitted via the fecal-oral route or via contaminated water or food and it may cause enteric infection in both humans and animals. The disease results in severe diarrhea and can be life threatening, particularly in very young and immunosuppressed individuals. More than 20** ***Cryptosporidium*** **species have been described infecting different vertebrates. Particularly,** ***Cryptosporidium*** **has been genetically characterized in 19 species of fish. Nevertheless, only few data are currently available regarding the characterization of** ***Cryptosporidium*** **species and genotypes in natural aquatic environments, and in particular in edible fish.**

Objective

Determine the prevalence of *Cryptosporidium* spp. in edible fish products in France (fresh water and marine fish)

Materials and methods

- Fish collection : 1512 fishes were collected during fishing sea campaigns along the French coasts, and from fisheries suppliers at Thonon-les-Bains at the border of the Geneva lake.
- Stomach and intestinal epithelial cells scrapping were scrapped.
- DNA extraction, detection and identification of *Cryptosporidium* by PCR-sequencing using degenerated primers targeting the 18S rRNA gene were performed.
- Subtype identification of *C. parvum* by amplification and sequencing of the GP60 gene.

Results

Figure 1. Prevalence of *Cryptosporidium* spp. in marine and fresh water fish

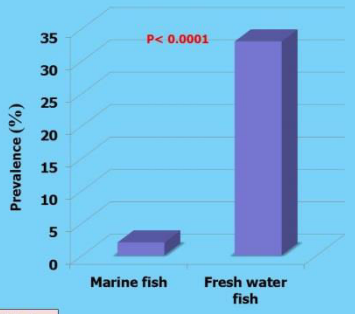


Figure 2. Distribution of *Cryptosporidium* spp. species

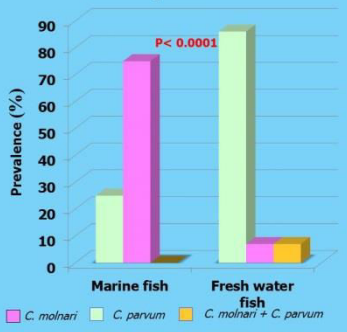


Table 1. Marine fish hosts

Fish common name	Latin name	No of individuals with *Cryptosporidium*
Saithe	*Pollachius_virens*	15
Cod	*Gadus_morhua*	1
Whiting	*Merlangius_merlangus*	1
Blue ling	*Molva dypterygia*	3
Atlantic mackerel	*Scomber_scombrus*	1
European pilchard	*Sardina pilchardus*	1
Chub mackerel	*Scomber japonicus*	2
Anchovy	*Engraulis encrasicoulus*	1
European hake	*Merluccius merluccius*	1
Atlantic herring	*Clupea harengus*	1
Common ling	*Molva_molva*	1

Figure 3. GP60 locus analysis of *C. parvum* (fresh water fish)

Subtypes:
IIaA15G2R1
IIaA17G2R1
IIaA16G2R1

Table 2. Fresh water fish hosts

Fish common name	Latin name	No of individuals with *Cryptosporidium*
Northern pike	*Esox lucius*	2
European whitefish	*Coregonus lavaretus*	4
European perch	*Perca fluviatilis*	4
Arctic char	*Salvelinus alpinus*	3
Common roach	*Rutilus rutilus*	1

Conclusions

- This is the first data about *Cryptosporidium* detection in fish in France.
- The overall frequency of *Cryptosporidium* spp. in sampled fishes from the Geneva lake was high (33%).The frequency of *Cryptosporidium* in wild marine fish was low (2%) but similar prevalences were reported in other studies in Australia and New Guinea.
- Two species of *Cryptosporidium* were detected infecting piscine hosts: *C. molnari* and *C. parvum.*
- The detected *C. parvum* subtypes have been commonly found infecting cattle and/or humans.
- 16 new species of fish hosts for *Cryptosporidium* were identified.
- Of importance to public health was the high rate of detection of *C. parvum* among piscine hosts, being this species the most common source of zoonotic infection.
- Fish may be a good sentinel for the detection of water contamination by sewage or agricultural run-off.

14ème Journée André VERBERT : 11 septembre 2014, Faculté de Médecine de Lille - pôle recherche, Lille, France.

5th International *Giardia* and *Cryptosporidium* Conference: 27-30 Mai 2014. Uppsala, Suède (Présentation orale)

4ème Forum Doctoral-EDST-UL: 18 Novembre 2014, Beyrouth, Liban;.

Prevalence, genetic diversity and risk factors of *Cryptosporidium* and *Giardia* infections among school children in Lebanon

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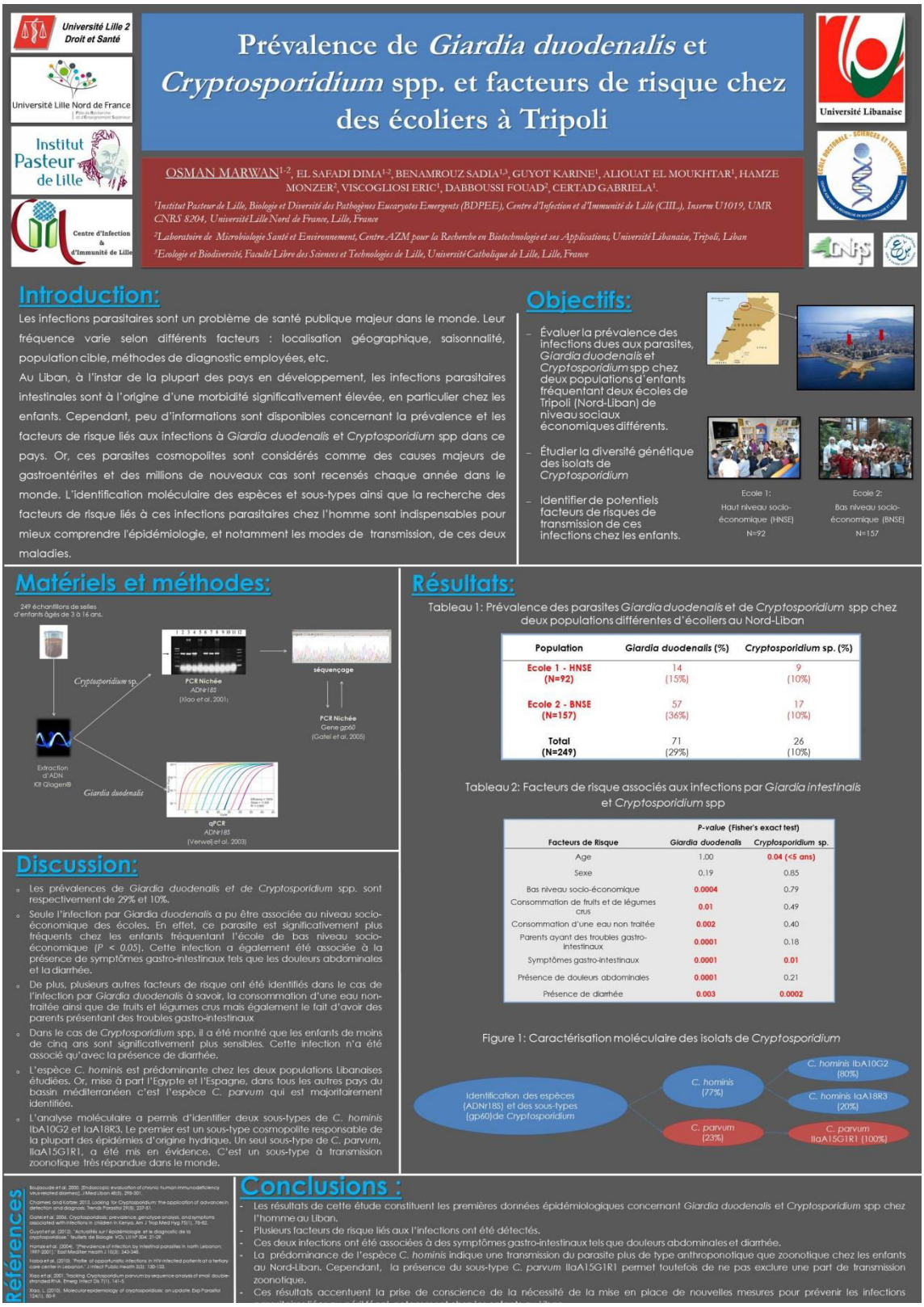
Abstract

Cryptosporidium and *Giardia* are protozoan enteroparasites with worldwide distribution that infect the gastrointestinal tract of vertebrates including humans. Both parasites have been recognized as the predominant cause of waterborne and foodborne outbreaks. They are among the major causative agents of gastroenteritis and nutritional disorders in humans, with millions of new cases occurring every year. *Cryptosporidium* oocysts and *Giardia* cysts are infectious immediately upon being excreted in feces and have the potential to be transmitted by fecal-oral route. Due to a common link with poverty especially in developing countries, both pathogens were included in the World Health Organization's Neglected Disease Initiative since 2004. The situation of *Cryptosporidium* and *Giardia* infections is not clear in Lebanon, a developing country often affected by parasitic infections. This study was devoted to determine the prevalence and the genetic diversity of *Cryptosporidium* and *Giardia*, in two children populations with different socio-economic level in Lebanon, as well as the risk factors associated with these two infections. Fecal samples obtained from children were examined microscopically by direct-light microscopy of wet mounts. In addition, modified

Ziehl-Neelsen staining as well as molecular tests were done for the detection of *Cryptosporidium* oocysts. Out of 250 children, *Giardia* and *Cryptosporidium* were present in 14.4% and 5.6% respectively according to microscopy examination. Based on molecular tools, the prevalence of cryptosporidiosis cases raised to 10% with predominance of C. hominis (74%). Subgenotype analysis of the isolates at the 60-kDa glycoprotein (GP60) locus identified two subtypes IbA10G2 (83%) and IaA18R3 (17%) for C. hominis and only one subtype IIaA15G1R1 for C. parvum (100%). The study of genetic diversity of *Giardia* is now in progress. Several risk factors such as age, low socio-economic status, eating raw vegetables and fruits, drinking non-treated water, having parents with gastro-intestinal symptoms and presence of abdominal pain were associated with *Giardia*sis. However, cryptosporidiosis was only correlated with having meals outside home and presence of gastro-intestinal symptoms especially diarrhea ($p < 0.05$). This work constitutes the first molecular epidemiology study outlining risk factors associated with cryptosporidiosis and *Giardia*sis in Lebanon.



Prévalence de *Giardia duodenalis* et *Cryptosporidium* spp. et facteurs de risque chez des écoliers à Tripoli

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Introduction:

Les infections parasitaires sont un problème de santé publique majeur dans le monde. Leur fréquence varie selon différents facteurs : localisation géographique, saisonnalité, population cible, méthodes de diagnostic employées, etc.

Au Liban, à l'instar de la plupart des pays en développement, les infections parasitaires intestinales sont à l'origine d'une morbidité significativement élevée, en particulier chez les enfants. Cependant, peu d'informations sont disponibles concernant la prévalence et les facteurs de risque liés aux infections à *Giardia duodenalis* et *Cryptosporidium* spp dans ce pays. Or, ces parasites cosmopolites sont considérés comme des causes majeurs de gastroentérites et des millions de nouveaux cas sont recensés chaque année dans le monde. L'identification moléculaire des espèces et sous-types ainsi que la recherche des facteurs de risque liés à ces infections parasitaires chez l'homme sont indispensables pour mieux comprendre l'épidémiologie, et notamment les modes de transmission, de ces deux maladies.

Objectifs:

- Évaluer la prévalence des infections dues aux parasites, *Giardia duodenalis* et *Cryptosporidium* spp chez deux populations d'enfants fréquentant deux écoles de Tripoli (Nord-Liban) de niveau sociaux économiques différents.
- Étudier la diversité génétique des isolats de *Cryptosporidium*
- Identifier de potentiels facteurs de risques de transmission de ces infections chez les enfants.

Ecole 1: Haut niveau socio-économique (HNSE) N=92

Ecole 2: Bas niveau socio-économique (BNSE) N=157

Matériels et méthodes:

249 échantillons de selles d'enfants âgés de 3 à 16 ans.

Cryptosporidium sp.

PCR Nichée ADNr18S (Xiao et al, 2001)

séquençage

PCR Nichée Gene gp60 (Gatel et al, 2005)

Extraction d'ADN Kit Qiagen®

Giardia duodenalis

qPCR ADNr18S (Verweij et al, 2003)

Résultats:

Tableau 1: Prévalence des parasites *Giardia duodenalis* et de *Cryptosporidium* spp chez deux populations différentes d'écoliers au Nord-Liban

Population	*Giardia duodenalis* (%)	*Cryptosporidium* sp. (%)
Ecole 1 - HNSE (N=92)	14 (15%)	9 (10%)
Ecole 2 - BNSE (N=157)	57 (36%)	17 (10%)
Total (N=249)	71 (29%)	26 (10%)

Tableau 2: Facteurs de risque associés aux infections par *Giardia intestinalis* et *Cryptosporidium* spp

	P-value (Fisher's exact test)	
Facteurs de Risque	*Giardia duodenalis*	*Cryptosporidium* sp.
Age	1.00	0.04 (<5 ans)
Sexe	0.19	0.85
Bas niveau socio-économique	0.0004	0.79
Consommation de fruits et de légumes crus	0.01	0.49
Consommation d'une eau non traitée	0.002	0.40
Parents ayant des troubles gastro-intestinaux	0.0001	0.18
Symptômes gastro-intestinaux	0.0001	0.01
Présence de douleurs abdominales	0.0001	0.21
Présence de diarrhée	0.003	0.0002

Figure 1: Caractérisation moléculaire des isolats de *Cryptosporidium*

Identification des espèces (ADNr18S) et des sous-types (gp60) de *Cryptosporidium*

C. hominis (77%) — *C. hominis* IbA10G2 (80%); *C. hominis* IaA18R3 (20%)

C. parvum (23%) — *C. parvum* IIaA15G1R1 (100%)

Discussion:

- Les prévalences de *Giardia duodenalis* et de *Cryptosporidium* spp. sont respectivement de 29% et 10%.
- Seule l'infection par Giardia *duodenalis* a pu être associée au niveau socio-économique des écoles. En effet, ce parasite est significativement plus fréquents chez les enfants fréquentant l'école de bas niveau socio-économique ($P < 0.05$). Cette infection a également été associée à la présence de symptômes gastro-intestinaux tels que les douleurs abdominales et la diarrhée.
- De plus, plusieurs autres facteurs de risque ont été identifiés dans le cas de l'infection par *Giardia duodenalis* à savoir, la consommation d'une eau non-traitée ainsi que de fruits et légumes crus mais également le fait d'avoir des parents présentant des troubles gastro-intestinaux
- Dans le cas de *Cryptosporidium* spp, il a été montré que les enfants de moins de cinq ans sont significativement plus sensibles. Cette infection n'a été associé qu'avec la présence de diarrhée.
- L'espèce *C. hominis* est prédominante chez les deux populations Libanaises étudiées. Or, mise à part l'Egypte et l'Espagne, dans tous les autres pays du bassin méditerranéen c'est l'espèce *C. parvum* qui est majoritairement identifiée.
- L'analyse moléculaire a permis d'identifier deux sous-types de *C. hominis* IbA10G2 et IaA18R3. Le premier est un sous-type cosmopolite responsable de la plupart des épidémies d'origine hydrique. Un seul sous-type de *C. parvum*, IIaA15G1R1, a été mis en évidence. C'est un sous-type à transmission zoonotique très répandue dans le monde.

Conclusions :

- Les résultats de cette étude constituent les premières données épidémiologiques concernant *Giardia duodenalis* et *Cryptosporidium* spp chez l'homme au Liban.
- Plusieurs facteurs de risque liés aux l'infections ont été détectés.
- Ces deux infections ont été associées à des symptômes gastro-intestinaux tels que douleurs abdominales et diarrhée.
- La prédominance de l'espèce *C. hominis* indique une transmission du parasite plus de type anthroponotique que zoonotique chez les enfants au Nord-Liban. Cependant, la présence du sous-type *C. parvum* IIaA15G1R1 permet toutefois de ne pas exclure une part de transmission zoonotique.
- Ces résultats accentuent la prise de conscience de la nécessité de la mise en place de nouvelles mesures pour prévenir les infections

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20th LAAS International Science Conference: 27-29 Mars 2014, Beyrouth, Lebanon.

Journées Franco-Maghrébines de Parasitologie et Mycologie: 23-26 Octobre 2013, Rabat, Maroc.

Epidémiologie moléculaire de la cryptosporidiose au Liban

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Le genre *Cryptosporidium* (Alveolata: Apicomplexa) comprend des espèces qui infectent le tractus gastro-intestinal ou respiratoire d'un grand nombre de vertébrés y compris l'homme. Ces espèces sont à l'origine d'une maladie opportuniste et cosmopolite, la cryptosporidiose. Celle-ci peut provoquer des diarrhées en général auto-résolutives chez les patients immunocompétents mais pouvant devenir chroniques, voire létales, chez les sujets immunodéprimés, notamment sidéens. Les espèces infectant le plus fréquemment l'homme sont *C. parvum,* infectant aussi les bovins et *C. hominis*. A l'instar de la plupart des pays en développement, le Liban est très touché par les parasitoses intestinales. Cependant, aucune information n'est disponible concernant la situation de la cryptosporidiose dans ce pays. Les études d'épidémiologie moléculaire ont souvent aidé les chercheurs à clarifier les facteurs de risque de transmission à l'homme de micro-organismes pathogènes. C'est pourquoi une étude a été menée visant à déterminer la prévalence et la nature de la diversité génétique du parasite chez l'homme et les bovins. Pour ce faire, un examen microscopique suivi d'une détection moléculaire par PCR nichée ciblant le gène de ---l'ARNr 18S a été réalisé sur des selles de patients et de bovins originaires du Nord-Liban. Cette étude a permis de mettre en évidence une prévalence de la cryptosporidiose de 9.2% chez des patients symptomatiques, de 10% chez des écoliers et de 7,8% chez des bovins. Deux espèces ont été identifiées dans cette population Libanaise : *C. hominis* (73% des isolats) et *C. parvum* (27%) alors que chez les bovins trois espèces ont été détectées : *C. andersoni* (50%), *C. bovis* (33%) et *C. parvum* (17%). L'étude du polymorphisme génétique des isolats de *C. hominis* et *C. parvum* réalisée

à l'aide du marqueur gp60, nous a permis d'identifier trois génotypes de *C. hominis* : IbA10G2, IaA18R3 et IdA19 chez l'homme et deux génotypes de *C. parvum,* IIaA15G1R1 et IIaA15G2R1 chez l'homme comme chez les bovins. Ces derniers sont des génotypes à transmission zoonotique très répandue dans le monde. Nous pouvons ainsi conclure que bien que la prédominance de l'espèce *C. hominis* laisse présager un mode de transmission plutôt anthroponotique, les résultats du génotypage ne permettent pas d'exclure une transmission zoonotique. Ces résultats constituent les premières données épidémiologiques concernant la cryptosporidiose au Liban.

Université Lille 2 Droit et Santé

Université Lille Nord de France

Institut Pasteur de Lille

Centre d'Infection & d'Immunité de Lille

EPIDÉMIOLOGIE MOLÉCULAIRE DE LA CRYPTOSPORIDIOSE AU LIBAN

Université Libanaise

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Introduction:

Les parasites du genre *Cryptosporidium* (*Alveolata : Apicomplexa*) sont des protozoaires intracellulaires qui infectent le tractus gastro-intestinal ou respiratoire d'un grand nombre de vertébrés, y compris l'homme. Ces parasites opportunistes sont à l'origine de la cryptosporidiose qui est une zoonose cosmopolite à transmission féco-orale. Cette infection est auto-résolutive chez les personnes immunocompétentes mais peut devenir chronique voir létale chez les personnes immunocompromises. Actuellement, 26 espèces de *Cryptosporidium* ont été validées. Les espèces infectant le plus fréquemment l'homme sont *Cryptosporidium parvum* (*C. parvum*) et *C. hominis* (Chalmers and Katzer, 2013). Ces protozoaires sont la cause de nombreuses épidémies d'origine hydrique et alimentaire. C'est pourquoi l'OMS a classé ce parasite en 2006 parmi les pathogènes émergents avec un fort impact en santé publique (WHO, 2006).

La cryptosporidiose est une infection cosmopolite avec une prévalence très variable selon les pays. La prévalence de l'infection dans la population humaine générale varie entre 1 et 3% dans les pays développés et peut aller jusqu'à 13% dans les pays en voie de développement. La mesure de la prévalence varie aussi en fonction de la méthode de diagnostique utilisée et de la population étudiée. La cryptosporidiose a également un impact sur la santé animale. Par exemple, chez les ruminants, un secteur important de l'économie agricole dans de nombreux pays, la cryptosporidiose est une cause bien connue de diarrhée néonatale pouvant être létale (Fayer and Santin, 2009).

Malgré sa pathogénicité, *Cryptosporidium* reste un parasite peu connu au Liban. Seule une étude rapporte une prévalence de 50%, chez des patients sidéens (Boujaoude et al, 2000). A l'heure actuelle, aucune information n'est disponible concernant la situation de la cryptosporidiose dans ce pays.

Objectifs:

- Évaluer la prévalence de la cryptosporidiose chez l'homme et les bovins au Liban.

- Étudier la diversité génétique des isolats de *Cryptosporidium*.

Population humaine totale

Patients diarrhéiques

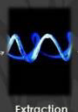
Bovins

Matériels et méthodes:

Echantillon de selles:

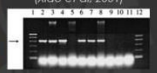
-225 Patients immunocompétents entre 1 et 86 ans (diarrhéiques ou non-diarrhéiques)

-154 Bovins



Extraction d'ADN
Kit Qiagen®

Nested PCR
Gène ARNr 18S
(*Xiao et al, 2001*)



Si *C. parvum* ou *C. hominis*: Nested PCR
Gène Gp60 (*Gafei et al. 2006*)

Séquençage

Identification des espèces de *Cryptosporidium* et des sous-types de *C. parvum* et *C. hominis*

Coloration de Ziehl-Neelsen modifiée
(*Henriksen et Pohlenz, 1981*)

Résultats:

Population humaine (N)0	Infection par *Cryptosporidium* (%)	Diarrhéiques infectés (%)	Non diarrhéiques infectés (%)
Totale (N=225)	18/225 (8%)	15/150 (10%)	3/75 (4%)
Enfants (N=119)	12/119 (10%)	9/62 (14.5%)	3/57 (5.2%)
Adultes (N=106)	6/106 (5.6%)	6/88 (6.8%)	0/18 (0%)

Identification des espèces de *Cryptosporidium* (Gène ARNr 18S)

C. hominis (67%)

C. parvum (33%)

C. andersoni (50%)

C. bovis (33%)

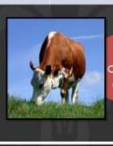
C. parvum (17%)

Identification des sous-types de *Cryptosporidium* (Gène gp60)

C. hominis IdA19 (100%)

C. parvum IIaA15G1R1 (80%) IIaA15G2R1 (20%)

C. parvum
IIaA15G1R1 (50%)
IIaA15G2R1 (50%)



7.8% de cryptosporidiose chez les bovins

Discussion:

Espèces prédominantes
- *C. parvum*
- *C. hominis*

Absence de données

Figure 1: Prévalence et distribution de la cryptosporidiose chez l'homme dans le bassin méditerranéen.

Absence de données

Figure 2: Prévalence et distribution de la cryptosporidiose chez les bovins dans le bassin méditerranéen.

Au Liban, la prévalence de la cryptosporidiose chez les sujets immunocompétents est dans la moyenne de ce qui a été décrit dans le bassin méditerranéen mais elle reste au dessus de ce qui a été décrit dans les pays développés (Figure 1). Les enfants semblent plus touchés que les adultes, comme le rapportent d'autres études notamment en France, aux USA et au Royaume Uni (Chalmers *et al*, 2011).

Par contre, la prévalence chez les bovins est similaire à celles décrites dans les pays développés (Figure 2).

L'espèce *C. parvum* est prédominante dans la plupart des pays du bassin méditerranéen. Cependant au Liban, comme en Egypte et en Espagne c'est l'espèce *C. hominis* qui prédomine.

Le génotypage nous a permis d'identifier un seul sous-type de *C. hominis* IdA19 chez l'homme. C'est un sous-type très rare qui n'a été décrit qu'au Canada (Trotz-Williams et al, 2006). Alors que deux sous-types de *C. parvum*, IIaA15G1R1 et IIaA15G2R1 ont été mis en évidence chez l'homme et les bovins. Ce sont des sous-types à transmission zoonotique très répandue dans le monde (Xiao *et al*, 2010).

Bien que la prédominance de l'espèce *C. hominis* laisse présager un mode de transmission plutôt anthroponotique, les résultats du génotypage ne permettent pas d'exclure une transmission zoonotique.

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Conclusions :

- Nos études ont permis de rapporter les premières données épidémiologiques sur la cryptosporidiose chez l'homme et les bovins au Liban.

- La cryptosporidiose semble être plus fréquente chez l'enfant que chez l'adulte.

- Les espèces *C. hominis* et *C. andersoni* sont prédominantes respectivement chez l'homme (67%) et les bovins (50%), ce qui laisse présager une contamination humaine plus anthroponotique que zoonotique au Liban.

- Les sous types *C. parvum* IIaA15G1R1 et *C. parvum* IIaA15G2R1 ont été identifiés chez l'homme et les bovins ce qui n'exclut pas une part de transmission zoonotique.

3ème Forum Doctoral-EDST-UL : 25 Juin 2013, Beyrouth, Liban.

Caractérisation génétique et phénotypique de *Cryptosporidium*: de la souris à l'homme

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Le genre *Cryptosporidium (Alveolata : Apicomplexa)* infecte le tractus gastro-intestinal d'un grand nombre de vertébrés, y compris l'homme chez qui il est responsable d'une maladie opportuniste à transmission féco-orale, la cryptosporidiose. Ce protiste est classé par l'OMS parmi les pathogènes émergents avec un fort impact en santé publique. Chez les sujets immunocompétents, l'infection se traduit par des diarrhées auto-résolutives qui peuvent devenir chroniques, voire létales, chez les sujets immunodéprimés. Les espèces infectant le plus fréquemment l'homme sont *Cryptosporidium parvum* (*C. parvum*) et *C. hominis*. De plus, notre laboratoire a montré (*Certad et al*, 2007, 2010, 2012) que trois souches de *C. parvum* (Iowa, TUM1 et Did) ont la capacité d'induire chez un modèle de souris SCID, traitée ou non par la dexaméthasone, une infection durable associée au développement d'adénocarcinomes digestifs invasifs. Par contre, *C. muris*, espèce pouvant également infecter l'homme et la souris SCID, n'induit pas ce type de transformation. Pour une meilleure compréhension de ce phénomène chez l'homme, mon projet de thèse associe une étude épidémiologique au nord du Liban et une étude de génomique comparative sur les génomes de *C. muris* et des trois souches de *C. parvum*. La première vise à étudier la diversité génétique et la transmission de *Cryptosporidium* spp. Ainsi que la mise en évidence d'une association entre la pathologie cancéreuse digestive et l'infection par *Cryptosporidium* chez l'homme. La seconde a quant à elle, pour but d'identifier les propriétés intrinsèques de *C. parvum* lui conférant ce pouvoir tumorigène. Dans un premier temps, un examen microscopique suivi d'une analyse moléculaire ont été réalisés sur des selles de patients symptomatiques. Cette étude a permis de mettre en évidence une prévalence de 9.2% de cryptosporidiose dans cette population et deux espèces ont pu être identifiées associées à l'infection, *C. hominis* (67%) et *C. parvum* (33%). Ce sont les premières données épidémiologiques concernant la cryptosporidiose au Liban.

Caractérisation génétique et phénotypique de Cryptosporidium: de la souris à l'homme
Marwan Osman[a,b], Sadia Benamrouz[b,c], Karine Guyot[b], Dima El Safadi[a,b], Monzer Hamze[a], Eduardo Dei-Cas[b], Eric Viscogliosi[b], El Moukhtar Alioua[b], Gabriela Certad[b], Fouad Dabboussi[a]
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Introduction
Objectifs
Axes de recherche
Diversité génétique et transmission de Cryptosporidium spp. dans la population libanaise
Liban
9.2%
Association entre Néoplasie digestive et le parasitisme par Cryptosporidium spp. chez l'homme
Génomique comparative sur les génomes de C. muris et trois souches de C. parvum (Iowa, TUM1 et Did)
CryptoDB
Conclusions
Perspectives
Références
Journées Scientifiques à l'Ecole Doctorale de Sciences et Technologie-2012/2013

2. Autres articles publiés dans des journaux internationaux

1. Article A: Short report: Molecular epidemiology of *Blastocystis* in Lebanon and correlation between subtype 1 and gastrointestinal symptoms

Dima El Safadi, Dionigia Meloni, Philippe Poirier, **Marwan Osman**, Amandine Cian, Lobna Gaayeb, Ivan Wawrzyniack, Frederic Delbac, Hicham El Alaoui, Laurence Delhaes, Eduardo Dei-Cas, Hassan Mallat, Fouad Dabboussi, Monzer Hamze, and Eric Viscogliosi*

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Abstract:

Blastocystis is the most common eukaryotic parasite in the intestinal tract of humans. Due to its potential impact in public health, we acquired the first data concerning the prevalence of this parasite and the frequency of the *Blastocystis* subtypes (STs) in the Lebanese population. In this study, fecal samples from 220 Lebanese symptomatic and asymptomatic patients were collected and a total of 42 patients (19%) were identified as positive for this parasite by direct-light microscopy of smears. Among these, 36 *Blastocystis* isolates were genotyped using partial small subunit ribosomal RNA gene sequencing. ST distribution in the present Lebanese population was as follows: ST3 (33.3%), ST2 (33.3%), ST1 (30.6%) and ST4 (2.8%). These data were compared to those available in other Middle Eastern and neighboring countries. Finally, ST1 was significantly more prevalent among symptomatic patients of this Lebanese population.

Manuscript:

Blastocystis is the most common intestinal parasite of humans and a wide range of animals with a worldwide distribution.[1] Its prevalence can reach 30% to 60% in developing countries and 1.5% to 20% in industrialized countries.[1] Even if the clinical significance of this parasite remains controversial, *Blastocystis* has been correlated with various gastrointestinal symptoms. It may also play a significant role in irritable bowel syndrome (IBS) and has been linked with urticaria.[2-6] According to recent *in vivo* and *in vitro* studies as well as genomic data, a model for pathogenesis of this parasite was proposed, mainly involving cysteine proteases secreted by the parasite.[5-7] *Blastocystis* organisms found in different hosts are morphologically indistinguishable. However, this genus exhibits an extensive genetic diversity and at least 13 subtypes (STs) have been described on the basis of molecular data[8-10], which showed sufficient genetic divergence to be classified as separate species.[11] Moreover, nine of these STs (ST1-ST9) have been isolated from human fecal samples highlighting both the low host specificity of the parasite and its zoonotic potential.[9-11] In the recent literature it is still in debate whether distinct *Blastocystis* STs correlate with the development of gastrointestinal symptoms caused by the parasite.[1,4,5,12,13] Moreover, information on the distribution of STs in some geographic locations including Middle Eastern countries is only starting to emerge. Therefore, the aim of the present study was to acquire the first epidemiological data regarding the prevalence of *Blastocystis* in the Lebanese population together with the frequency of STs in symptomatic and asymptomatic patients.

To conduct this study, fecal specimens were randomly collected at six hospitals in North Lebanon (Nini Hospital, Governmental Hospital of Tripoli, Tripoli Center for Medical Analysis, Hamidi Medical Center, Monla Hospital and Saydet Zgharta Hospital) from 220 patients living in or in the vicinity of Tripoli during the period of March-April 2011. These patients were followed up for different pathologies such as gastrointestinal symptoms or presented for routine medical checkups. Stool samples were subsequently examined by direct-light microscopy of smears (DLM) for the presence of *Blastocystis* at the Centre AZM of Tripoli. No information was available on potential viral or bacterial infections. Genomic DNA was directly extracted from fecal samples positive for *Blastocystis* by DLM using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Each sample was amplified by non-quantitative PCR (non-qPCR) as previously described using two independent pairs of *Blastocystis*-specific primers[14,15], both targeting the small subunit (SSU) rDNA coding region. The respective 600 bp and 520 bp-amplified domains have been shown to provide sufficient sequence information to discriminate between *Blastocystis* STs.[14,15] For each DNA sample, the non-qPCR product with the highest intensity on agarose gel was purified and cloned as previously described.[16] Two clones containing inserts of approximately the expected size were arbitrarily selected for each sample and sequenced. DNA samples negative by non-qPCR were subsequently amplified using the highly sensitive real-time quantitative PCR (qPCR) assay developed by Poirier and others.[17] The expected 320 bp-amplified variable region of the SSU rRNA gene was directly sequenced for subtyping. To compare the subtyping data obtained by molecular methods, 7 DNA samples were amplified by both non-qPCR and qPCR methods. The SSU rRNA gene sequences obtained in this study have been deposited in GenBank under accession numbers KC294143 to KC294196. These new sequences were aligned with the use of the BioEdit v7.0.1 package (http://www.mbio.ncsu.edu:BioEdit/bioedit.html) then compared with all the *Blastocystis* SSU rRNA gene sequences available from the NCBI using the BLAST program. Subtypes were identified by determining the closest similarity against all known *Blastocystis* STs according to the last classification by Stensvold and others.[8]

A total of 42 patients (19%) were positive for *Blastocystis* by DLM. This high prevalence was in the same range as those observed in other neighboring countries such as Egypt and Iran.[18,19] It could roughly reflect the overall carriage rate of the parasite in the Lebanese population because most patients included in the present study were followed up for various pathologies other than intestinal or were asymptomatic. However, the prevalence

of *Blastocystis* in our Lebanese population was more likely underestimated since several authors[15,17] pointed out the poor sensitivity of DLM compared to either non-qPCR or qPCR assays. This strongly suggested that the actual prevalence of *Blastocystis* in Lebanon might be much higher making this parasite a potential major problem in public health.

Among the 42 positive samples by DLM, 6 were unsuccessfully amplified by either non-qPCR or qPCR due probably to the presence of known PCR inhibitors in fecal samples. The remaining 36 isolates were collected from 15 females and 21 males, ranging in age from 1 to 83 years (Table 1). The symptomatic group consisted of 19 patients presenting variously with diarrhea, abdominal pain, vomiting, constipation, some in association with fatigue and fever. As previously reported[1,5], abdominal pain and diarrhea were the two major symptoms among *Blastocystis*-positive Lebanese patients. The asymptomatic group was comprised of 17 individuals without any gastrointestinal symptoms. Each of the SSU rDNA gene sequences obtained from the 36 isolates showed 98% to 100% identity to representative sequences of *Blastocystis* STs reported so far, allowing the direct subtyping of these isolates (Table 1). For 8 of the 19 positive samples for which a 600 bp or 520 bp- fragment of the SSU rRNA gene was cloned, the two sequenced clones were identical (Table 1). Clones showed one to four nucleotide differences for 10 of the remaining samples that could be explained by sequence variations between SSU rDNA gene copies within the same isolate.[7,16,20] For the last sample DS25 (ST3), there were 10 nucleotide differences between both clones suggesting a possible coinfection of the patient with two variants within the same ST. In this regard, substantial intra-ST diversity has been recently demonstrated in ST3.[20] The 7 samples amplified by non-qPCR and qPCR yielded identical subtyping results.

All the 36 samples genotyped in this study represented single infections. As shown in Table 1, ST3 (33.3%) and ST2 (33.3%) were the most common in our Lebanese population followed by ST1 (30.6%) and ST4 (2.8%). In most countries around the world, a majority of human *Blastocystis* infections were attributable to ST3 isolates.[16] This was also the case in the Lebanese population even if the frequencies of ST1 and ST2 were identical or roughly similar to that of ST3. The ST distribution in Lebanon can now be compared to those of other Middle Eastern countries such as Iran[19] and to neighboring countries like Turkey[21,22] and Egypt.[23,24] In these countries, ST1 was the second most common variant after ST3 while it follows at third position in Lebanon but still has a high frequency. ST2 was globally poorly represented in Iran and Egypt whereas it was commonly found in Turkey and Lebanon. In addition, in our Lebanese population only 1/36 isolates has been genotyped as ST4. This ST

has not been found in Egypt and Iran and was only identified in a single patient in Turkey. Overall ST4 is common in Europe[25] and much less frequent or absent in other geographical regions. In summary, our data showed a prevalence of ST1, ST2, and ST3 and a virtual absence of ST4 in the Middle Eastern and neighboring countries as well as some geographic variation in the frequency of ST2 that might reflect different exposure to animal and/or environmental infection sources.

To evaluate the pathogenic potential of the different *Blastocystis* STs in our Lebanese population, the phylogenetic distribution of the 36 genotyped isolates from symptomatic and asymptomatic individuals was examined. ST3 (8 of 12 isolates) and ST2 (7/12) were dominant in the asymptomatic group reinforcing the hypothesis that most isolates of these subtypes are likely to be non pathogenic.[5] The single ST4 isolate of our study was asymptomatic while ST4 has been shown to be common in patients with acute diarrhea.[26] Strikingly 10/11 ST1 isolates composed the symptomatic group and a statistical analysis done with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) using the Fisher's exact test showed a significant association between ST1 and gastrointestinal symptoms (p=0.0113). Recently, epidemiological surveys have reported the frequency of STs from symptomatic and asymptomatic individuals in China,[27] Turkey,[28] and Iran[19] and showed that ST1 was over-represented in groups of symptomatic patients. Moreover ST1 was the most prevalent ST of *Blastocystis* in patients with IBS[24,29] and human ST1 isolates were associated with elevated pathogenicity in experimentally infected rats.[30] However, patient symptomatic status was uncorrelated with *Blastocystis* ST and symptoms in the context of several other epidemiological studies.[16,21,31,32]

To our knowledge this is the first investigation of prevalence and molecular epidemiology of *Blastocystis* in Lebanon. In this country, the prevalence of this parasite would be high with predominance of ST3, ST2 and ST1 isolates. A consistent link between ST1 and gastrointestinal symptoms was identified and should be confirmed in further studies including a larger number of patients.

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TABLE 1

Clinical data and *Blastocystis* subtypes among symptomatic and asymptomatic patients in Lebanon

Patients	Sex/age	Symptoms	*Blastocystis* ST by non-qPCR*	Nucleotide differences†	*Blastocystis* ST by qPCR*	Accession no.
DS1	M/71	Diarrhea			1	KC294143
DS2	M/65				3	KC294144
DS3	F/10	Abdominal pain Vomiting			2	KC294145
DS4	M/4	Diarrhea Vomiting Fever			1	KC294146
DS5	M/69	Diarrhea Abdominal pain			1	KC294147
DS6	M/33				2	KC294148
DS7	M/44				1	KC294149
DS8	M/21	Diarrhea Abdominal pain Vomiting			1	KC294150
DS9	M/12	Diarrhea Vomiting Fever	2 (Sc)	4	2	KC294151-3

DS10	M/13	Diarrhea Abdominal pain Fever Fatigue	1 (Sc)	2	1	KC294154-6
DS11	F/27	Diarrhea	2 (Sc)	0		KC294157
DS12	F/23		3 (Sc)	0	3	KC294158-9
DS13	M/60		3 (Sc)	0	3	KC294160-1
DS14	F/30	Abdominal pain	3 (Sc)	0	3	KC294162-3
DS15	M/34	Abdominal pain			3	KC294164
DS16	M/40				2	KC294165
DS17	M/29	Diarrhea Abdominal pain			1	KC294166
DS18	F/6				2	KC294167
DS19	F/31	Abdominal pain	2 (Sc)	0		KC294168
DS20	F/5		3 (St)	1		KC294169-70
DS21	M/51				2	KC294171
DS22	F/83				2	KC294172
DS23	F/20	Abdominal pain Fatigue Constipation	1 (St)	3	1	KC294173-5
DS24	M/3	Diarrhea Abdominal pain			1	KC294176

		Vomiting				
DS25	M/24		3 (Sc)	10		KC294177-8
DS26	F/11	Diarrhea	3 (Sc)	1		KC294179-80
		Abdominal pain				
DS27	F/5	Abdominal pain	1 (St)	1		KC294181-82
		Vomiting				
		Fatigue				
DS28	F/8		4 (Sc)	0		KC294183
DS29	M/8		2 (Sc)	0		KC294184
DS30	M/5	Abdominal pain			2	KC294185
DS31	M/16	Abdominal pain	1 (Sc)	2		KC294186-7
DS32	F/23		3 (St)	1		KC294188-9
DS33	F/22				2	KC294190
DS34	F/40		3 (Sc)	2		KC294191-2
DS35	M/35		3 (St)	0		KC294193
DS36	M/1	Diarrhea	3 (St)	2	3	KC294194-6
		Abdominal pain				

*According to the new standard terminology[8]; (St) and (Sc): non-qPCR using the primer pair described by Stensvold and others[15] and Scicluna and others[14], respectively

†Determined in the common region of two clones sequenced for each sample

2. Article B: Acute *Blastocystis*-Associated Appendicular Peritonitis in a Child, Casablanca, Morocco

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Abstract:

Despite increasing reports that *Blastocystis* infection is associated with digestive symptoms, its pathogenicity remains controversial. We report appendicular peritonitis in a 9-year-old girl returning to France from Morocco. Only *Blastocystis* parasites were detected in stools, appendix, peritoneal liquid, and recto-uterine pouch. Simultaneous gastroenteritis in 26 members of the child's family suggested an outbreak.

Manuscript:

Blastocystis is a genus of anaerobic protozoan parasites that infect humans and a vast range of animal species. Prevalence in humans varies from 0.5%–24% in industrialized countries to 30%–76% in developing countries (1, 2). Classic clinical features of infection include gastrointestinal symptoms such as nausea, anorexia, flatulence, and acute or chronic diarrhea. Fever is usually absent. An association with irritable bowel syndrome and extraintestinal manifestations such as urticaria has been suggested (2). Reports about invasive infection or disseminated diseases are rare (3). Here, we report the case of a pediatric patient infected with *Blastocystis* that was manifested by gastroenteritis associated with suppurative appendicitis and peritonitis.

In August 2013, a 9-year-old girl who was returning to France after a 1-month stay with her family in Casablanca, Morocco, was admitted to Lille University Hospital in Lille. Symptoms started in Casablanca 3 days before hospital admission and included fever, severe diarrhea (>10 liquid defecations/day), vomiting, and abdominal pain in the hypogastric area and in the right and left lower quadrants associated with bilateral dorsal pain, anorexia, and weakness.

Blood count showed 13,850/mm3 leukocytes (75.4% neutrophils, 15.9% lymphocytes, 8.5% monocytes). C-reactive protein level was increased at 266 mg/L (Low risk: <1.0mg/L; average risk: 1.0–3.0 mg/L; high risk >3.0 mg/L). Traveler's gastroenteritis was diagnosed, and symptomatic treatment with acetaminophen, phloroglucinol glucoside, and acetorphan was prescribed. However, abdominal pain increased, and total food intolerance occurred in the following hours.

An abdominal ultrasound was performed, revealing appendicitis with suppuration in the recto-uterine pouch and a reflex ileus. Parasitologic examination of fecal matter revealed only abundant *Blastocystis* vacuolar forms, with >5 parasites per field. We further confirmed absence of Cryptosporidium spp. using glycerin assay and real-time PCR. Yeasts and a multimicrobial flora were present in the fecal material, but other infectious agents such as

Salmonella spp., Shigella spp., Campylobacter spp., Yersinia enterocolitica, adenovirus, and rotavirus were not detected. Similarly, multimicrobial flora, but no pathogenic bacteria, were detected in the peritoneal liquid and recto-uterine pouch (Table). Histopathologic observation revealed acute suppurative appendicitis with ulcerations extending deep into the muscularis, which was covered with a suppurative and fibrinous exudate. We observed infiltration by numerous neutrophils, eosinophils, plasma cells, and lymphocytes through all layers and into the serous membrane (Figure, panel A).

After hematoxylin-eosin staining and immunofluorescence labeling by using the anti-*Blastocystis* Paraflor B monoclonal antibody (Boulder Diagnostics, Marlborough, MA, USA), we detected parasitic forms in the lumen and in the lamina propria of the mucosa (Figure, panels B, C). We used real-time PCR for *Blastocystis* parasite detection as described (4), using DNA extracted from stools, appendix, peritoneal liquid, and the recto-uterine pouch, which all tested positive. We subsequently performed small subunit rDNA (SSU rDNA) amplification, then cloned the PCR product and sequenced 5 clones from all the DNA samples to subtype (ST) *Blastocystis* isolates and detect mixed infections (5). We identified ST3 in all the analyzed compartments. Mixed infection with ST2 and ST3 was detected only in the stools. The SSU rDNA gene sequences obtained in this study have been deposited in GenBank under accession nos. KJ605630–KJ605649.

The child completely recovered after an appendectomy, removal of a stercolith from the appendix lumen, and treatment with tinidazole, 20 mg/kg/d, and ceftriaxone, 50 mg/kg/d for 10 days, together with gentamicin, 5 mg/kg/d for 5 days. Although tinidazole is not the first line medication for treatment of *Blastocystis* infection, the child recovered completely and showed total clearance of parasites at day 73: using microscopy and real-time PCR on fecal samples, we found negative results for *Blastocystis*. Data obtained from the child's mother revealed simultaneous cases of gastroenteritis in 26 family members: 13 adults, 34–98 years of age, and 13 children, 18 months–15 years of age, who lived in the same building at the residential "Mohammadi" area of Casablanca. Adults had mild or moderate diarrhea but symptoms were more severe in children, who all had abundant diarrhea, vomiting, and weight loss. Repatriation in France of an 18-month-old baby was considered, but his condition improved. None of the family members required hospitalization. Unfortunately, no explorations were performed, and the diagnosis could not be microbiologically documented.

Conclusions

Reports of *Blastocystis* infection associated with diarrhea and clinical symptoms in immunocompetent and immunocompromised patients have increased during the past 2 decades (2). Tissue invasion of *Blastocystis* parasites in the appendix (6) or in the colon mucosa (3), associated with acute or chronic inflammation, has been reported. However, although controversy still exists over whether this parasite is commensal or pathogenic, this case supports its invasive and inflammatory potential. Previous reports regarding the presence of *Blastocystis* parasites in 4 of 100 appendix specimens from patients with acute appendicitis (7), and of pseudoappendicular illness, which led to appendectomies in children with intestinal infection caused by this parasite (8), suggest that *Blastocystis* infection could be associated with appendicitis. Nevertheless, the actual role of *Blastocystis* in the pathogenesis of appendicitis remains inconclusive. In this report, the presence of a stercolith, which can be found in 50%–80% of appendicitis cases, suggested mechanical obstruction of the appendix's lumen, which is the main etiology of appendicitis.

Here, we report dissemination of *Blastocystis* into the lumen, the mucosa, and the recto-uterine pouch exudate, associated with appendicular acute inflammation, and no other infectious agent was detected. These observations, together with the well-documented acute or chronic inflammation occurring in humans or animals with *Blastocystis* infections (3,9), likely support the contribution of this infection to the inflammatory process. Infection with ST3 further reinforced this hypothesis. Indeed, the presence of pathogenic strains among ST3 has been confirmed through experimental infections in rats (9). Additionally, a substantial inflammatory reaction and an increased propagation of human colorectal cancer cells exposed to *Blastocystis* ST3 antigens has been demonstrated in vitro (10). For humans, the pathogenicity of different STs is unclear and remains a debatable issue. ST1 isolates were found to be more prevalent among symptomatic patients in Lebanon (5), but ST3 was found to be the only ST that showed pathogenic potential in Malaysian patients when compared with ST1 and ST2 (11). ST3 was also found to be significantly associated with diarrhea in Libya ($p = 0.008$) (12). For this case, the fact that only ST3 was detected in all analyzed samples, whereas a mixed infection with ST2 and ST3 was found in the child's stools, further supports the high invasive potential of ST3. ST3 is the most common ST in Europe, but, in African countries, its frequency varies from 17.8% in Libya (12) to 61.9% in Egypt (13). In Morocco, a 28.7% prevalence of blastocystosis has been reported, but data concerning the ST distribution of the parasite are not available (14). Furthermore, although *Blastocystis*

infection could not be confirmed among the child's relatives, the simultaneous occurrence of gastroenteritis cases in the same family and the absence of other infectious agents in the child's stools suggest a potential outbreak of *Blastocystis* infection. *Blastocystis* parasites could have spread within the child's family, as previously reported in Italy, where 2 adopted children originating from India and the Côte d'Ivoire transmitted *Blastocystis* parasites to their adoptive parents and grandmother (15). Possible acquisition of this parasite from a common source such as contaminated water could also explain family transmission in this report. Altogether, these data highlight 1) the need for both systematic parasitologic examinations of stools in patients with invasive infections who are traveling from countries with high *Blastocystis* prevalence and 2) the need for routine provision of imidazoles for empiric treatment of peritonitis.

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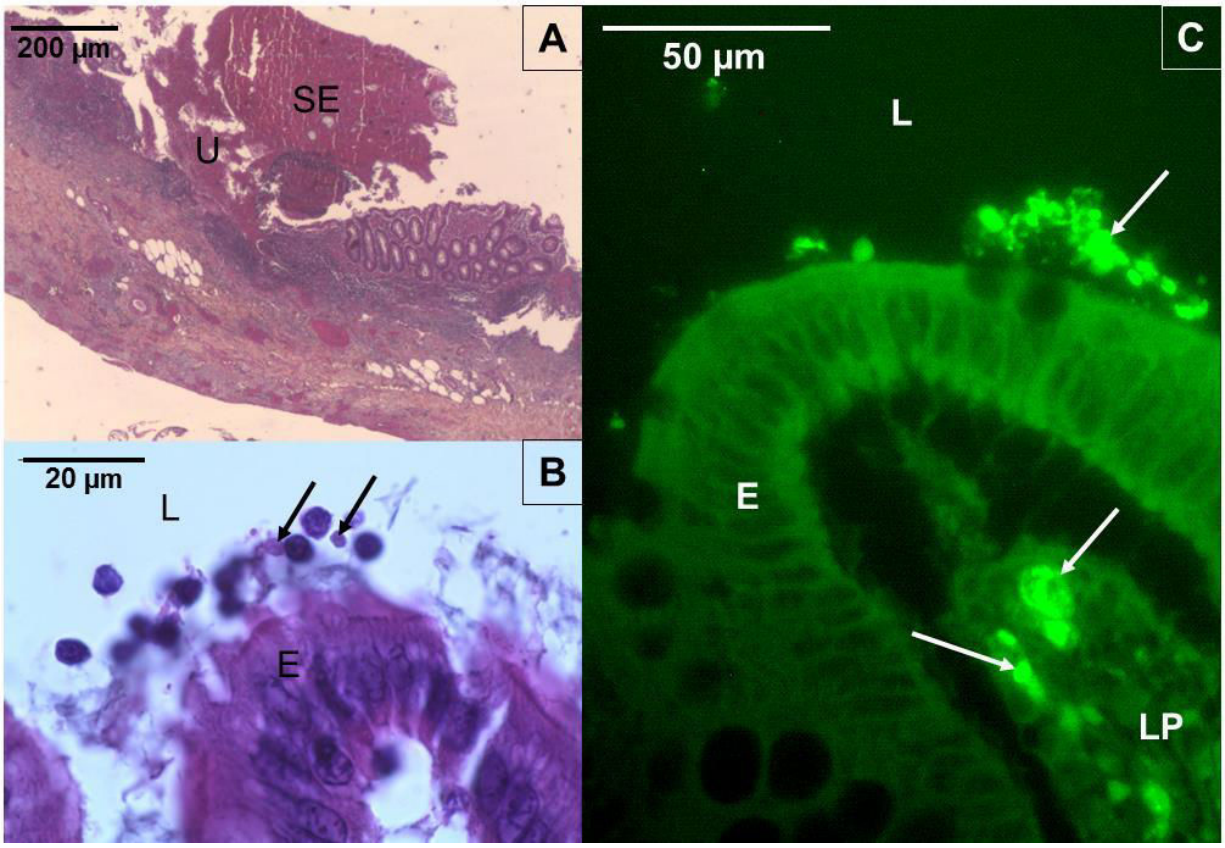
Table. Microbiological examination of samples of feces, appendix, recto-uterine pouch, and peritoneal fluid from child who had peritonitis, Casablanca, Morocco*

Variable	Date of sampling, 2013				
	Aug 30	Sept 1			Nov 13
Sample type	Feces	Appendix	Recto-uterine pouch	Peritoneal fluid	Feces
Microscopy result	Numerous *Blastocystis* vacuolar forms; no *Cryptosporidium* or other parasites	Presence of rare *Blastocystis*†	ND	ND	Absence of parasites
Real-time PCR result *Blastocystis* spp. *Cryptosporidium* spp. *Blastocystis* spp. genotype	 Positive Negative ST2, ST3	 Positive Negative ST3	 Positive Negative ST3	 Positive Negative ST3	 Negative ND ND
Bacterial culture result	Negative for: *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia enterocolitica*	ND	Multimicrobial flora	Multimicrobial flora	ND
Viral antigen detection	Negative: aenovirus and rotavirus	ND	ND	ND	ND

*ND, not done; ST, sequence type.

†Figure, panels B,C.

Figure. Histopathologic examination of appendix samples from a child who had peritonitis, Casablanca, Morocco, 2013. A) Ulceration (U) covered with suppurative and fibrinous exudates (SE) (hematoxylin-eosin). B) *Blastocystis* parasites (arrows) in the lumen (L), and at the surface of the epithelium (E) (hematoxylin-eosin). C) *Blastocystis* parasites (arrows) in the lumen, at the surface of the epithelium and in the lamina propria (LP) of the mucosa (immunofluorescence labeling with anti-*Blastocystis* ParaFlorB antibody).



3. Article C: Draft genome sequence of the intestinal parasite *Blastocystis* subtype 4-isolate WR1

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Abstract

The intestinal protistan parasite *Blastocystis* is characterized by an extensive genetic variability with 17 subtypes (ST1-ST17) described to date. Only the whole genome of a human ST7 isolate was previously sequenced. Here we report the draft genome sequence of *Blastocystis* ST4-WR1 isolated from a laboratory rodent at Singapore.

Keywords

Blastocystis subtype 4-isolate WR1, Illumina-HiSeq, Whole genome, Annotation using maker gene annotation pipeline

Specifications

Organism/cell line/tissue	*Blastocystis* ST4
Strain	WR1
Sequencer or array type	Illumina-HiSeq 2000
Data format	Processed
Experimental factors	Laboratory rodent and cultured axenically
Experimental features	Draft genome sequence of the intestinal parasite *Blastocystis* ST4-WR1 isolate
Consent	N/A
Sample source location	Clermont-Ferrand, France

Direct link to data

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JPUL02000000 (http://www.ncbi.nlm.nih.gov/nuccore/JPUL00000000.2)

Manuscript:

Experimental Design, Materials and Methods

The stramenopile *Blastocystis* is a common anaerobic protist living in the digestive tract of several animal groups (1). Its prevalence in human often exceeds 5% in industrialized countries (1) and can reach 100% in developing countries (2). Although the role of *Blastocystis* as a human pathogen remains unclear, it has been associated with acute or chronic digestive disorders and some epidemiological surveys have suggested an association

with Irritable Bowel Syndrome (IBS) (3, 4). In patients with IBS, *Blastocystis* seems to be associated with a decrease of the fecal microbiota protective bacteria, *Bifidobacterium* sp. And *Faecalibacterium prausnitzii* (5). The life cycle of the parasite is poorly documented. Among the parasitic forms described in the literature, the vacuolar stage which is maintained *in vitro* in axenic culture, is the most easily recognizable and the most frequently observed in stool samples. *Blastocystis* exhibits an extensive genetic diversity and seventeen subtypes (ST1-ST17) have been identified based on the gene coding for the small-subunit ribosomal RNA (6) among which the first nine are found in humans. The whole genome of a human *Blastocystis* ST7 isolate was previously sequenced. Briefly, it consists of an 18.8 Mbp nuclear genome with 6,020 predicted genes (7) and a circular genome of 29 kbp (8) located within mitochondria-like organelles (MLO). Other MLO genomes with conserved gene synteny have also been sequenced from *Blastocystis* ST1, ST3 and ST4 isolates (9, 10). Here we report the sequencing of the *Blastocystis* ST4-WR1 genome from an isolate of a laboratory rodent and cultured axenically (11). Genomic DNA was isolated using a Qiagen DNeasy blood and tissue kit and sequencing was performed with the Illumina HiSeq 2000 system (Genoscreen, Lille, France). A total of 43.855.085 of 100-bp high quality paired-end reads were generated and were *de novo* assembled using the IDBA-ud algorithm (12). The output was then scaffolded using SSPACE (13) and gaps were filled by Gapfiller software (14). In total, 1,301 scaffolds from 494 bp to 133,271 bp were obtained, with a scaffold N50 of 29,931 bp. The draft genome sequence of *Blastocystis* ST4 has a deduced total length of 12.91 Mbp and a G+C content of 39.7%. Assembly also provided a circular DNA molecule of 27,717 bp in size with a G+C content of 21.9% corresponding to the whole MLO genome sequence. Genes were carried out using the Maker gene annotation pipeline (15). The Maker pipeline was set with the results of *ab initio* gene prediction algorithms Augustus (16) and SNAP (17), the 6,020 protein-coding genes of *Blastocystis* ST7 (5), ESTs of both *Blastocystis* ST7 (5) and ST1 (18) and 414 manually-designed genes of the ST4-WR1 isolate. Basic information about the assembled genome and predicted genes are shown in Table 1. Gene functions were annotated by BLAST2GO (19) and BLAST analyses with NCBI (http://www.ncbi.nlm.nih.gov/). 183 tRNA were predicted using tRNAscan-SE 1.21 (20). The preliminary annotation data revealed that *Blastocystis* ST4-WR1 nuclear genome harbors 5,720 protein-coding genes. The presence of proteases was determined using BLAST against MEROPS database (21), and secreted proteases were identified using SIGNALP 3.0 (22) and WoLF PSORT (23). Finally, OrthoMCL (24) was applied to compare both ST4 and ST7

genomes. This comparative analysis revealed that the ST4 genome contains less duplicated genes than ST7 and that more than 30% of ST4 genes have no ortholog in the ST7 genome at an *E* value cutoff of 10-5. This also led to the identification of new candidate genes, in particular some potential virulence factors, including 20 secreted proteases that may be involved in the physiopathology of this parasite. Among these proteases, 7 seem to be specific to ST4 as no ortholog has been found in the ST7 genome. Sequencing and annotation of additional ST (ST1, ST2, ST3 and ST8) genomes are under progress and should be helpful for a better understanding of the genetic diversity, pathogenesis, metabolic potential and genome evolution of this highly prevalent human parasite.

Table 1. Genome statistics and intron features of *Blastocystis* ST4 and ST7

	***Blastocystis* ST4**	***Blastocystis* ST7**
Genome assembly size	12.91 Mbp	18.8 Mbp
G + C content	39.6%	45.2%
Number of genes	5,720	6,021
Average gene size	1,386 bp	1,299 bp
Genes with introns	92.7%	84.6%
Average exon number per gene	5.06	4.58
Average length of introns (nt number)	33	50
Average length of proteins (aa number)	416	359
MLO genome size	27,815 bp	29,270 bp
MLO G +C content	21.94%	20.03%
Number of MLO genes	45	45

Conflict of interest

Authors declare no conflict of interest.

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